(54) Title: COMPOSITIONS AND METHODS FOR INHIBITION OF HIV-1 INFECTION

(57) Abstract: This invention provides a composition which comprises an admixture of three compounds, wherein: a) one compound is an antibody which binds to a CCR5 receptor; b) one compound retards attachment of HIV-1 to a CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell; and c) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of any two of the compounds in the admixture ranges form about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ CELL. This invention also ———  hibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with an a invention effective to inhibit HIV-1 infection of the CD4+ cell so as to thereby inhibit HIV-

# COMPOSITIONS AND METHODS FOR INHIBITION OF HIV-1 INFECTION

This application is a continuation and claims the benefit of PCT International Application No. PCT/US01/02633, filed January 26, 2001, U.S. Provisional Application No. 60/266,738, filed February 6, 2001, U.S. Serial No. 09/912,824, filed July 25, 2001; and U.S. Serial No. 09/663,219, filed September 15, 2000, the contents of which are hereby incorporated by reference into this application.

Throughout this application, various publications are referenced within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found immediately preceding the claims.

## Background of the Invention

Infection of cells by human immunodeficiency virus type 1 (HIV-1) is mediated by the viral envelope (env) glycoproteins gp120 and gp41, which are expressed as a noncovalent, oligomeric complex on the surface of virus and virally infected cells. HIV-1 entry into target cells proceeds at the cell surface through a cascade of events

that include (1) binding of the viral surface glycoprotein gp120 to cell surface CD4, which is the primary receptor for HIV-1, (2) env binding to fusion coreceptors such as CCR5 and CXCR4, and (3) multiple conformational changes in gp41. During fusion, gp41 adopts transient conformations that include a prehairpin fusion intermediate that ultimately folds into a conformation capable of mediating fusion. These events culminate in fusion of the viral and cellular membranes and the subsequent introduction of the viral genome into the target cell. A similar sequence of molecular events is required for infection to spread via fusion of infected and uninfected cells. Each stage of the viral entry process can be targeted for therapeutic intervention.

HIV-1 attachment can be inhibited both by agents that bind the viral envelope glycoproteins and by agents that bind human CD4. Notably, HIV-1 attachment can be inhibited by compounds that incorporate the gp120-binding domains of human CD4 and molecular mimics thereof [1-7]. Because this interaction between gp120 and CD4 is essential for virus infection, CD4-based molecules have the potential to target most if not all strains of HIV-1. In addition, viruses have limited ability to develop resistance to such molecules.

The determinants for gp120 binding map to the first extracellular domain (D1) on CD4 [1], and the amino acids critical for binding center on a loop comprising amino

acids 36-47. Potent HIV-1 inhibitory activity has been reproduced in a 27-amino acid peptide that mimics this loop and surrounding structures [7].

5 A number of recombinant CD4-based molecules have been developed and tested for clinical activity in man. The first of these contained the four extracellular domains (D1-D4) of CD4 but lacked the transmembrane and intracellular regions. This molecule, termed soluble CD4

10 (sCD4), demonstrated excellent tolerability when administered to humans at doses ranging to 10 mg/kg [8,9]. Transient reductions in plasma levels of infectious HIV-1 were observed in certain patients treated with sCD4. The short half-life of sCD4 in humans (45 minutes following

15 intravenous administration) was identified as one obstacle to using this agent for chronic therapy.

Second-generation CD4-based proteins were developed with increased serum half-life. These CD4-immunoglobulin fusion

20 proteins comprised the D1D2 domains of CD4 genetically fused to the hinge CH2 and CH3 regions of human IgG molecules. These divalent proteins derive HIV-1 neutralizing capacity from their CD4 domains and Fc effector functions from the IgG molecule. A CD4-IgG1 fusion

25 protein was shown to have excellent tolerability and improved pharmacokinetics in Phase I clinical testing [10]. The antiviral evaluations were inconclusive.

More recently, a third-generation tetravalent CD4-IgG2

fusion protein was developed that comprises the D1D2 domains of CD4 genetically fused to the heavy and light chain constant regions of human IgG2. This agent binds the HIV-1 envelope glycoprotein gp120 with nanomolar affinity [5] and may inhibit virus attachment both by receptor blockade and by detaching gp120 from the virion surface, thereby irreversibly inactivating the virus. In standard PBMC-based neutralization assays, CD4-IgG2 neutralized primary HIV-1 isolates derived from all major subtypes and outlier groups. The CD4-IgG2 concentrations required to achieve a 90% reduction in viral infectivity, the in vitro IC90, were approximately 15-20 $\mu$g/ml [11], concentrations that are readily achievable in vivo. CD4-IgG2 was similarly effective in neutralizing HIV-1 obtained directly from the plasma of seropositive donors in an ex vivo assay, indicating that this agent is active against the diverse viral quasispecies that are encountered clinically [12]. CD4-IgG2 also provided protection against infection by primary isolates in the hu-PBL-SCID mouse model of HIV-1 infection [13]. Recent analyses have demonstrated that CD4-IgG2's ability to neutralize primary viruses is independent of their coreceptor usage [14].

Compared with mono- or divalent CD4-based proteins, CD4-IgG2 has consistently demonstrated as much as 100-fold greater potency at inhibiting primary HIV-1 isolates [5,12,14,15]. The heightened potency may derive from CD4-IgG2's ability to bind virions with increased valency/avidity and its steric juxtaposition of two gp120

binding sites on each Fab-like arm of the immunoglobulin molecule. The larger Fab-like arms of CD4-IgG2 are also more likely to span HIV-1 envelope spikes on the virion. In a variety of preclinical models, CD4-IgG2's anti-HIV-1 activity has been shown to compare favorably with those of the rare human monoclonal antibodies that broadly and potently neutralize primary HIV-1 isolates [5,11,14,15]. In addition, CD4-IgG2 therapy is in principle less susceptible to the development of drug-resistant viruses than therapies employing anti-env monoclonal antibodies or portions of the highly mutable HIV-1 envelope glycoproteins. These properties suggest that CD4-IgG2 may have clinical utility as an agent that neutralizes cell-free virus before it has the opportunity to establish new rounds of infection. In addition to treatment, CD4-IgG2 may have utility in preventing infection resulting from occupational, perinatal or other exposure to HIV-1.

In Phase I clinical testing, single-dose CD4-IgG2 demonstrated excellent pharmacology and tolerability. In addition, measurable antiviral activity was observed by each of two measures. First, a statistically significant acute reduction in plasma HIV RNA was observed following administration of a single 10 mg/kg dose. In addition, sustained reductions in plasma levels of infectious HIV were observed in each of two patients tested. Taken together, these observations indicate that CD4-IgG2 possesses antiviral activity in humans [16].

In addition to CD4-based proteins and molecular mimics thereof, HIV-1 attachment can also be inhibited by antibodies and nonpeptidyl molecules. Known inhibitors include (1) anti-env antibodies such as IgG1b12 and F105 [17,18], (2) anti-CD4 antibodies such as OKT4A, Leu 3a, and humanized versions thereof [19,20], and (3) nonpeptidyl agents that target either gp120 or CD4 [21], [22-24]. The latter group of compounds includes aurintricarboxylic acids, polyhydroxycarboxylates, sulfonic acid polymers, and dextran sulfates.

Several agents have been identified that block HIV-1 infection by targeting gp41 fusion intermediates. These inhibitors may interact with the fusion intermediates and prevent them from folding into final fusogenic conformations. The first such agents to be identified comprised synthetic or recombinant peptides corresponding to portions of the gp41 ectodomain predicted to form hydrophobic alpha helices. One such region is present in both the amino and carboxy segments of the extracellular portion of gp41, and recent crystallographic evidence suggests that these regions interact in the presumed fusogenic conformation of gp41 [25,26]. HIV-1 infection can be inhibited by agents that bind to either N- or C-terminal gp41 epitopes that are exposed during fusion. These agents include the gp41-based peptides T-20 (formerly known as DP178), T-1249, DP107, N34, C28, and various fusion proteins and analogues thereof [27-33]. Other studies have identified inhibitors that comprise non-natural D-peptides

and nonpeptidyl moieties [34,35]. Clinical proof-of-concept for this class of inhibitors has been provided by T-20, which reduced plasma HIV RNA levels by as much as 2 logs in Phase I/II human clinical testing [36]. The broad antiviral activity demonstrated for this class of inhibitors reflects the high degree of gp41 sequence conservation amongst diverse strains of HIV-1.

Recent studies [37] have demonstrated the possibility of raising antibodies against HIV-1 fusion intermediates. This work employed "fusion-competent" HIV vaccine immunogens that capture transient fusion intermediates formed upon interaction of gp120/gp41 with CD4 and fusion coreceptors. The immunogens used in these studies were formalin-fixed cocultures of cells that express HIV-1 gp120/gp41 and cells that express human CD4 and CCR5 but not CXCR4. The antibodies elicited by the vaccines demonstrated unprecedented breadth and potency in inhibiting primary HIV-1 isolates regardless of their coreceptor usage, indicating that the antibodies were raised against structures such as gp41 fusion intermediates that are highly conserved and transiently exposed during HIV-1 entry. This class of antibodies does not include the anti-gp41 monoclonal antibody known as 2F5, which interacts with an epitope that is constitutively presented on virus particles prior to fusion [38].

Previously, synergistic inhibition of HIV-1 entry has been demonstrated using certain anti-env antibodies used in

combination with other anti-env antibodies [39-44], anti-CD4 antibodies [45], or CD4-based proteins [6]. Similarly, synergies have been observed using anti-CCR5 antibodies used in combination with other anti-CCR5 antibodies, CC-chemokines, or CD4-based proteins [46]. Our prior studies described in U.S. Serial No. 09/493,346 examined combinations of fusion inhibitors and attachment inhibitors. Our prior studies described in PCT International Application No. PCT/US99/30345, WO 00/35409, published June 22, 2000 examined combinations of HIV-1 attachment inhibitors and CCR5 coreceptor inhibitors. However, no prior studies have examined the combination of fusion inhibitors and CCR5 coreceptor inhibitors, nor the triple combination of fusion inhibitors, CCR5 coreceptor inhibitors and HIV-1 attachment inhibitors.

## Summary of the Invention

This invention provides a composition which comprises an admixture of two compounds, wherein: (a) one compound is an antibody or portion thereof which binds to a CCR5 receptor; and (b) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit

HIV-1 infection of the CD4+ cell.

This invention provides a composition which comprises an admixture of three compounds, wherein: (a) one compound is an antibody or portion thereof which binds to a CCR5 receptor; (b) one compound retards attachment of HIV-1 to a CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell; and (c) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of any two of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with an amount of the composition of the subject invention effective to inhibit HIV-1 infection of the CD4+ cell so as to thereby inhibit HIV-1 infection of the CD4+ cell.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (1) an amount of an antibody which binds to a CCR5 receptor and (2) an amount of a compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding

noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (1) an amount of an antibody which binds to a CCR5 receptor, (2) an amount of a compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell effective to inhibit HIV-1 infection of the CD4+ cell, and (3) an amount of a compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

## Brief Description of the Figures

### Figure 1

Synergistic inhibition of HIV-1 entry

CD4-IgG2 (--■--), T-20 (--●--), and a 25:1 CD4-IgG2:T-20 combination (···▲···) were analyzed for inhibition of HIV-1 entry in an env-mediated membrane fusion (RET) assay. Inhibitors were added to a mix of HeLa-Env$_{JR-FL}$⁺ and PM1 cells previously labeled with F18 and R18 respectively. Fluorescence RET was measured after 4h of incubation, and percent inhibition was calculated as described [19]. Results are mean values from three independent experiments. The data were analyzed according to the median effect principle described in Equation (1). The best-fit parameters for K and m are 0.31 $\mu$g/ml and 0.73 for CD4-IgG2, 0.017 $\mu$g/ml and 0.92 for T-20, and 0.11 $\mu$g/ml and 1.0 for their combination. These curves are plotted and indicate a reasonable goodness-of-fit between experiment and theory ($r^2$ = 0.983, 0.998, and 0.996 for CD4-IgG2, T-20, and their combination, respectively). To normalize for the differences in potencies of the compounds, separate concentrations scales are used for CD4-IgG2 and the 25:1 CD4-IgG2:T-20 mixture and for T-20, as indicated.

### Figure 2

Combination indices for inhibition of HIV-1 entry by combinations of CD4-IgG2 and T-20. CD4-IgG2, T-20 and fixed mass ratios thereof were analyzed in the RET assay for the ability to inhibit env-mediated membrane fusion.

The 25:1 (high) combination examined 10 three-fold serial dilutions of 250 $\mu$g/ml CD4-IgG2, 10 $\mu$g/ml T-20 and their combination. The 25:1 (low) combination examined 10 three-fold serial dilutions of 50 $\mu$g/ml CD4-IgG2, 2 $\mu$g/ml T-20 and their combination. The 5:1 combination examined 10 three-fold serial dilutions of 50 $\mu$g/ml CD4-IgG2, 2 $\mu$g/ml T-20, and their combination. The 1:1 combination examined 10 three-fold serial dilutions of 10 $\mu$g/ml CD4-IgG2, 10 $\mu$g/ml T-20 and their combination. Inhibition data from three or more independent assays were averaged prior to analysis. Dose-response curves for the various inhibitors and combinations were fit to Equation (1), which was then rearranged to calculate the inhibitor concentrations required to effect a given percent inhibition. The concentrations of the individual agents in an inhibitory mixture were calculated from their known mass ratios. These values were then used to calculate the Combination Index (CI) according to Equation (2). CI < 1 indicates synergy, CI = 1 indicates additive effects, and CI > 1 indicates antagonism.

Figure 3

Dose reductions observed for synergistic combinations of CD4-IgG2 and T-20. CD4-IgG2, T-20 and a 25:1 fixed mass ratio thereof were tested in the RET assay for the ability to inhibit env-mediated membrane fusion. Inhibition data from six independent assays were averaged. K and m were determined by curve-fitting the dose-response curves, and Equation (1) was rearranged to allow calculation of c for

a given f for the single agents and their combination. Dose Reduction is the ratio of the inhibitor concentrations required to achieve a given degree of inhibition when the inhibitor is used alone v. in a synergistic combination.

Figure 4

Dose reductions observed for combinations of CD4-IgG2, PRO 140, PA12 and T-20. The agents were tested individually and in combination for the ability to inhibit HIV env-mediated membrane fusion in the RET assay. a.) CD4-IgG2, PA12, T-20 and a ~1:1:10 fixed molar ratio thereof. b.) CD4-IgG2, PRO 140, T-20 and a ~2:1:20 fixed molar ratio thereof, c.) CD4-IgG2, PRO 140, T-20 and a ~4:1:30 fixed molar ratio thereof, and d.) PRO 140, T-20 and a 1:30 fixed molar ratio thereof where Dose Reduction is the ratio of the inhibitor concentrations required to achieve a given degree of inhibition when the inhibitor is used alone v. in a synergistic combination. 6-8 three-fold serial dilutions of a.) 125 nM CD4-IgG2, 167 nM PA12, 1100 nM T-20 and their combination, b.) 125 nM CD4-IgG2, 67 nM PRO 140, 1100 nM T-20 and their combination, c.) 125 nM CD4-IgG2, 33 nM PRO 140, 1100 nM T-20 and their combination, and d.) 36 nM PRO 140, 1100 nM T-20 and their combination were examined. The inhibitor concentrations required to effect a given percent inhibition were calculated. The concentrations of the individual agents in an inhibitory mixture were calculated from their known molar ratios. These values were then used to calculate the Combination Index (CI) according to Equation (2). CI < 1 indicates synergy, CI = 1 indicates

additive effects, and CI> 1 indicates antagonism.


Figure 5:

Triple combination of PRO 542, PRO 140 and T-20
Synergistically Blocks HIV-1 Entry. PRO 542, PRO 140 and T-
20 were used alone and in ~3:1:30 molar combination to
inhibit HIV-1$_{JR-FL}$ env-mediated cell-cell fusion. The
methodology for this assay is described in Litwin et al.
(67).


Figure 6

Synergistic inhibition of HIV-1 entry
CD4-IgG2 (--■--), T-20 (--●--), and a 25:1 CD4-IgG2:T-20
combination (···▲···) were analyzed for inhibition of HIV-1
entry in an env-mediated membrane fusion (RET) assay.
Inhibitors were added to a mix of HeLa-Env$_{JR-FL}$⁺ and PM1 cells
previously labeled with F18 and R18 respectively.
Fluorescence RET was measured after 4h of incubation, and
percent inhibition was calculated as described [19].
Results are mean values from three independent experiments.
The data were analyzed according to the median effect
principle described in Equation (1). The best-fit
parameters for K and m are 0.31 $\mu$g/ml and 0.73 for CD4-
IgG2, 0.017 $\mu$g/ml and 0.92 for T-20, and 0.11 $\mu$g/ml and 1.0
for their combination. These curves are plotted and
indicate a reasonable goodness-of-fit between experiment
and theory ($r^2$ = 0.983, 0.998, and 0.996 for CD4-IgG2, T-20,
and their combination, respectively). To normalize for the
differences in potencies of the compounds, separate

concentrations scales are used for CD4-IgG2 and the 25:1 CD4-IgG2:T-20 mixture and for T-20, as indicated.

Figure 7

Combination indices for inhibition of HIV-1 entry by combinations of CD4-IgG2 and T-20. CD4-IgG2, T-20 and fixed mass ratios thereof were analyzed in the RET assay for the ability to inhibit env-mediated membrane fusion. The 25:1 (high) combination examined 10 three-fold serial dilutions of 250 µg/ml CD4-IgG2, 10 µg/ml T-20 and their combination. The 25:1 (low) combination examined 10 three-fold serial dilutions of 50 µg/ml CD4-IgG2, 2 µg/ml T-20 and their combination. The 5:1 combination examined 10 three-fold serial dilutions of 50 µg/ml CD4-IgG2, 2 µg/ml T-20, and their combination. The 1:1 combination examined 10 three-fold serial dilutions of 10 µg/ml CD4-IgG2, 10 µg/ml T-20 and their combination. Inhibition data from three or more independent assays were averaged prior to analysis. Dose-response curves for the various inhibitors and combinations were fit to Equation (1), which was then rearranged to calculate the inhibitor concentrations required to effect a given percent inhibition. The concentrations of the individual agents in an inhibitory mixture were calculated from their known mass ratios. These values were then used to calculate the Combination Index (CI) according to Equation (2). CI < 1 indicates synergy, CI = 1 indicates additive effects, and CI > 1 indicates antagonism.

<u>Figure 8</u>

Dose reductions observed for synergistic combinations of
CD4-IgG2 and T-20. CD4-IgG2, T-20 and a 25:1 fixed mass
ratio thereof were tested in the RET assay for the ability
5    to inhibit env-mediated membrane fusion. Inhibition data
from six independent assays were averaged. K and m were
determined by curve-fitting the dose-response curves, and
Equation (1) was rearranged to allow calculation of c for
a given f for the single agents and their combination. Dose
10   Reduction is the ratio of the inhibitor concentrations
required to achieve a given degree of inhibition when the
inhibitor is used alone v. in a synergistic combination.

<u>Figure 9</u>

15   Inhibition of HIV-1$_{JR-FL}$ virus-cell fusion (A) and cell-cell
fusion (B-D) with PRO 542, T-20 and combinations thereof.
The PRO 542:T-20 molar ratio was 1:2 (A and C) or 1:10 (B).
The concentrations of PRO 542 and T-20 in the mixtures are
plotted for the purposes of illustrating dose reductions (A
20   and B) or lack thereof (C). In the washout assay (C), the
drugs were pre-incubated with HeLa-Env cells for 2 h at 25
°C prior to washing, warming and addition of PM1 cells. In
the cell-cell preincubation assay (D), HeLa-Env and PM1
cells were co-incubated for 2 h at 25 °C prior to the
25   addition of drugs and warming to 37 °C. The values
represent the averages of independent assays performed in
triplicate (A,B) or duplicate (C,D).

<u>Figure 10</u>

Combination index values and dose reductions for inhibition if HIV-1 entry with combinations of PRO542 and T-20.

Figure 11

5      HIV-1 entry involves at least three steps that provide targets for therapy: gp120-CD4 attachment; gp120-coreceptor interactions; gp41-mediated fusion.

Figure 12

10     PRO 542(CD4-IgG2) attachment inhibitor.

Figure 13

PRO 140 coreceptor inhibitor

15     Figure 14

T20 fusion inhibitor

Figure 15

HIV-1 virus cell fusion assay

20

Figure 16

Synergistic Inhibition of virus-cell fusion with PRO542 and T-20. PRO542 alone (···▲···), T-20 alone (--●--), 1:2 mix (--■--); (------) PRO 542 in mix; (ı ı ı ı ı ı) T-20 in mix. The

25     virus used was HIV-1JR-FL.

Figure 17

Synergistic Inhibition of virus-cell fusion with PRO542 and T-20. PRO542 and T-20 were used in a 1:2 molar ratio

Figure 18

HIV-1 Cell-Cell Fusion Assay. Resonance Energy Transfer (RET) assay of HIV-1 membrane fusion.

5    Figure 19

Synergistic Inhibition of cell-cell fusion with PRO542 and T-20. PRO542 alone (···▲···), T-20 alone (--●--), 1:10 mix (--■--); (------) PRO 542 in mix; (ιιιιιι) T-20 in mix. The virus used was HIV-1JR-FL.

10

Figure 20

Synergistic Inhibition of cell-cell fusion with PRO542 and T-20 in 1:2, 1:10 and 1:50 ratios.

15    Figure 21

PRO140, PRO542 and T-20 Triple Combination Synergistically Blocks HIV-1 Entry. PRO140 alone (···◆···), PRO542 alone (···▲···), T-20 alone (--●--), 1:3:30 mix (--■--); (___ ___ ___ ___ ___) PRO 140 in mix; (ιιιιιι) PRO542 in mix; (------- -) T-20 in mix.

20

Figure 22

PRO140, PRO542 and T-20 Triple Combination Synergistically Blocks HIV-1 Entry. PRO140, PRO542 and T-20 used in a

25    1:3:30 molar ratio.

Figure 23

PRO542 does not potentiate T-20 activity in the absence of coreceptor. PRO542 alone (···▲···), T-20 alone (--●--),

PRO542:T-20 cocktail (--■--); (-------) PRO542 in cocktail.


Figure 24

5    Formation of prehairpin intermediate requires CD4, coreceptor and 37°C. (--●--) T-20 standard assay; (--○--) T-20, 25°C preincubation; (···▲···) PRO542 standard assay; (--△--) PRO542, 25°C preincubation;


10    Figure 25

Formation of prehairpin intermediate requires CD4, coreceptor and 37°C. (--●--) T-20 standard assay; (--o--) T-20 temperature shift washout assay.


15    Figure 26

Possible mechanism of synergy: PRO542 increases the half-life of the T-20 targets.


Figure 27

20    Possible mechanism of synergy: PRO542 increases the half-life of the T-20 targets.


25


Figure 28

Synergistic inhibition of HIV-1 entry: PRO 542, T-1249, and

a 1:10 molar combination (4:1 mass combination) of PRO
542:T-1249 were analyzed for inhibition of HIV-1 entry in
an env-mediated membrane fusion (RET) assay. Inhibitors
were added to a mix of HeLa-EnvJR-FL$^+$ and PM1 cells
previously labeled with F18 and R18 respectively.
Fluorescence RET was measured after 4 hours of incubation,
and percent inhibition was calculated as described in
Litwin, et al. (J. Virol. 70: 6437, 1996). The
concentrations of PRO 542 and T-1249 present in the
cocktail are derived from the curve fit of the data and are
shown for the purposes of illustrating dose reductions.

## Figure 29

Combination indices and dose reductions observed for a 1:10
molar combination of PRO 542 and T-1249. Compounds were
analyzed as described in Figure 28. Combination Index
values were calculated according to the median effect
principle. Dose Reduction is the ratio of the inhibitor
concentrations required to achieve a given degree of
inhibition when the inhibitor is used alone verses in
combination.

## Detailed Description of the Invention

The plasmids CD4-IgG2-HC-pRcCMV and CD4-kLC-pRcCMV were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International

5      Recognition of the Deposit of Microorganisms (the "Budapest Treaty") for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 10801 University Blvd, Manassas, VA 20110-2209 under ATCC Accession Nos. 75193 and 75194, respectively. The plasmids were deposited

10     with ATCC on Jan. 30, 1992. The plasmid designated pMA243 was similarly deposited in accordance with the Budapest Treaty with ATCC under Accession No. 75626 on December 16, 1993.

15     The murine hybridomas PA8, PA9, PA10, PA11, PA12 and PA14 were deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (the "Budapest Treaty") for the Purposes of Patent Procedure with the

20     American Type Culture Collection (ATCC), 10801 University Blvd, Manassas, VA 20110-2209 under the following ATCC Accession Nos.: PA8 (ATCC No. HB-12605), PA9 (ATCC No. HB-12606), PA10 (ATCC No. HB-12607), PA11 (ATCC No. HB-12608), PA12 (ATCC No. HB-12609) and PA14 (ATCC No. HB-12610). The

25     hybridomas were deposited on December 2, 1998.

As used herein, the following standard abbreviations are used throughout the specification to indicate specific amino acids:

A=ala=alanine                 R=arg=arginine

N=asn=asparagine              D=asp=aspartic acid

C=cys=cysteine                Q=gln=glutamine

E=glu=glutamic acid           G=gly=glycine

5    H=his=histidine               I=ile=isoleucine

L=leu=leucine                 K=lys=lysine

M=met=methionine              F=phe=phenylalanine

P=pro=proline                 S=ser=serine

T=thr=threonine               W=trp=tryptophan

10   Y=tyr=tyrosine                V=val=valine

This invention provides a composition which comprises an admixture of two compounds, wherein: (a) one compound is an antibody or portion thereof which binds to a CCR5 receptor;

15   and (b) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of the compounds in the admixture ranges from about 100:1 to

20   about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

This invention provides a composition which comprises an admixture of three compounds, wherein: (a) one compound is

25   an antibody or portion thereof which binds to a CCR5 receptor; (b) one compound retards attachment of HIV-1 to a CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell; and (c) one compound retards gp41 from adopting a conformation

capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of any two of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

This invention provides a composition which comprises an admixture of two compounds, wherein one compound retards attachment of HIV-1 to a CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell and the other compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate, wherein the relative mass ratio of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

As used herein, "HIV-1" means the human immunodeficiency virus type-1. HIV-1 includes but is not limited to extracellular virus particles and the forms of HIV-1 associated with HIV-1 infected cells. HIV-1$_{JR-FL}$ is a strain that was originally isolated at autopsy from the brain tissue of an AIDS patient [47]. The virus was co-cultured with lectin-stimulated normal human peripheral blood mononuclear cells. The virus has been cloned and the DNA sequences of its envelope glycoproteins are known (Genbank Accession #U63632). In terms of sensitivity to inhibitors

of viral entry, HIV-1$_{JR-FL}$ is known to be highly representative of primary HIV-1 isolates [11,14,15,48-50].

As used herein, "gp41 fusion intermediates" includes
5    structures, conformations, and oligomeric states that are preferentially and transiently presented or exposed on the HIV-1 envelope glycoprotein gp41 during the process of HIV-1 env-mediated membrane fusion. These intermediates may form upon interaction of HIV-1 with cellular receptors or
10   may be present in partially or fully occluded states on HIV-1 prior to its interaction with cellular receptors. "gp41 fusion intermediates" do not include fusogenic gp41 conformations that cannot provide targets for therapeutic intervention.

15

The gp41 fusion intermediates may contain multiple epitopes that are transiently exposed during fusion and can provide targets for therapeutic intervention. As used herein, an "N-terminal gp41 epitope" may comprise all or portions of
20   the sequences from amino acid A541 to Q590. As used herein, a "C-terminal gp41 epitope" may comprise all or portions of the sequences from amino acid W628 to L663. These epitopes have the potential to form coiled-coils of interacting alpha helical segments by virtue of heptad
25   (sequence of seven amino acids) repeats containing hydrophobic amino acids at positions 1 and 4 of the heptad. The amino acid numbering system is for the HxB2 isolate of HIV-1 (Genbank Protein Accession No. AAB50262). Because of the sequence variability of HIV-1 envelope proteins, the

composition, size and precise location of such sequences may be different for different viral isolates. The gp41 fusion intermediates may also present other linear or conformational epitopes that are transiently expressed during HIV-1 entry. An inhibitor may target multiple epitopes present on gp41 fusion intermediates. Alternatively, separate inhibitors may be used in combination to target one or more epitopes present on gp41 fusion intermediates.

As used herein, "fusogenic" means capable of mediating membrane fusion. As used herein, "HIV-1 fusion coreceptor" means a cellular receptor that mediates fusion between the target cell expressing the receptor and HIV-1 or an HIV-1 envelope glycoprotein⁺ cell. HIV-1 fusion co-receptors include but are not limited to CCR5, CXCR4 and other chemokine receptors. As used herein, "fusion" means the joining or union of the lipid bilayer membranes found on mammalian cells or viruses such as HIV-1. This process is distinguished from the attachment of HIV-1 to a target cell. Attachment is mediated by the binding of the HIV-1 exterior glycoprotein to the human CD4 receptor, which is not a fusion co-receptor.

As used herein, "retards" means that the amount is reduced. As used herein, "attachment" means the process that is mediated by the binding of the HIV-1 envelope glycoprotein to the human CD4 receptor, which is not a fusion co-receptor. As used herein, "CD4" means the mature, native,

membrane-bound CD4 protein comprising a cytoplasmic domain, a hydrophobic transmembrane domain, and an extracellular domain which binds to the HIV-1 gp120 envelope glycoprotein.

As used herein, "epitope" means a portion of a molecule or molecules that form a surface for binding antibodies or other compounds. The epitope may comprise contiguous or noncontiguous amino acids, carbohydrate or other nonpeptidyl moities or oligomer-specific surfaces.

The compounds of the subject invention have shown to demonstrate a synergistic effect. As used herein, "synergistic" means that the combined effect of the compounds when used in combination is greater than their additive effects when used individually.

In one embodiment of the composition of this invention, the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a CD4-based protein.

As used herein, "CD4-based protein" means any protein comprising at least one sequence of amino acid residues corresponding to that portion of CD4 which is required for CD4 to form a complex with the HIV-1 gp120 envelope glycoprotein.

In one embodiment the CD4-based protein is a CD4-

immunoglobulin fusion protein. In one embodiment the CD4-immunoglobulin fusion protein is CD4-IgG2, wherein the CD4-IgG2 comprises two heavy chains and two lights chains, wherein the heavy chains are encoded by an expression vector designated CD4-IgG2HC-pRcCMV (ATCC Accession No. 75193) and the light chains are encoded by an expression vector designated CD4-kLC-pRcCMV (ATCC Accession No. 75194). As used herein, CD4-IgG2 is also referred to as PRO 542.

In one embodiment of the composition of this invention, the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a protein, the amino acid sequence of which comprises that of a protein found in HIV-1 as an envelope glycoprotein. In one embodiment, the protein binds to an epitope of CD4 on the surface of the CD4+ cell. In one embodiment the envelope glycoprotein is selected from the group consisting of gp120, gp160, and gp140.

In one embodiment of the composition of this invention, the compound which retards the attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is an antibody or portion of an antibody. In one embodiment, the antibody is a monoclonal antibody. In one embodiment, the monoclonal antibody is a human, humanized or chimeric

antibody. In one embodiment, the portion of the antibody is a Fab fragment of the antibody. In one embodiment, the portion of the antibody comprises the variable domain of the antibody. In one embodiment, the portion of the antibody comprises a CDR portion of the antibody. In one embodiment, the monoclonal antibody is an IgG, IgM, IgD, IgA, or IgE monoclonal antibody.

As used herein, "antibody" means an immunoglobulin molecule comprising two heavy chains and two light chains and which recognizes an antigen. The immunoglobulin molecule may derive from any of the commonly known classes, including but not limited to IgA, secretory IgA, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4. It includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or nonhuman antibody. A nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man. Methods for humanizing antibodies are known to those skilled in the art.

This invention provides humanized forms of the above antibodies. As used herein, "humanized" describes

antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody would retain a similar antigenic specificity as the original antibody, i.e., in the present invention, the ability to bind CCR5.

One skilled in the art would know how to make the humanized antibodies of the subject invention. Various publications, several of which are hereby incorporated by reference into this application, also describe how to make humanized antibodies. For example, the methods described in United States Patent No. 4,816,567 (58) comprise the production of chimeric antibodies having a variable region of one antibody and a constant region of another antibody.

United States Patent No. 5,225,539 (59) describes another approach for the production of a humanized antibody. This patent describes the use of recombinant DNA technology to

produce a humanized antibody wherein the CDRs of a variable region of one immunoglobulin are replaced with the CDRs from an immunoglobulin with a different specificity such that the humanized antibody would recognize the desired target but would not be recognized in a significant way by the human subject's immune system. Specifically, site directed mutagenesis is used to graft the CDRs onto the framework.

Other approaches for humanizing an antibody are described in United States Patent Nos. 5,585,089 (60) and 5,693,761 (61) and WO 90/07861 which describe methods for producing humanized immunoglobulins. These have one or more CDRs and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. These patents describe a method to increase the affinity of an antibody for the desired antigen. Some amino acids in the framework are chosen to be the same as the amino acids at those positions in the donor rather than in the acceptor. Specifically, these patents describe the preparation of a humanized antibody that binds to a receptor by combining the CDRs of a mouse monoclonal antibody with human immunoglobulin framework and constant regions. Human framework regions can be chosen to maximize homology with the mouse sequence. A computer model can be used to identify amino acids in the framework region which are likely to interact with the CDRs or the specific antigen and then mouse amino acids can be used at these positions to create the humanized antibody.

The above patents 5,585,089 and 5,693,761, and WO 90/07861 (62) also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid reside at the framework positions at which the amino acid is predicted to have a side chain atom within 3Å of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies.

In one embodiment, the monoclonal antibody binds to an HIV-1 envelope glycoprotein. In one embodiment, the HIV-1 envelope glycoprotein is selected from the group consisting of gp120 and gp160.

In one embodiment, the HIV-1 envelope glycoprotein is gp120

and the monoclonal antibody which binds to gp120 is IgG1b12 or F105. IgG1b12 is listed as item #2640 in the NIH AIDS Research and Reference Reagent Program Catalog. F105 is listed as item #857 in the NIH AIDS Research and Reference Reagent Program Catalog.

In one embodiment, the antibody binds to an epitope of CD4 on the surface of the CD4+ cell.

In one embodiment of the composition of this invention, the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a peptide. In one embodiment of the composition of this invention, the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a nonpeptidyl agent. As used herein, "nonpeptidyl" means that the agent does not consist in its entirety of a linear sequence of amino acids linked by peptide bonds. A nonpeptidyl agent may, however, contain one or more peptide bonds.

In one embodiment of the composition of this invention, the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is an antibody. In one embodiment the antibody is a monoclonal antibody. In one embodiment, the antibody is a polyclonal antibody.

In one embodiment of the composition of this invention, the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a peptide.

In one embodiment of the composition of this invention, the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a fusion protein which comprises a peptide which inlcudes but is not limited to T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ ID NO: 4), and N34(L6)C28 (SEQ ID NO: 5). In one embodiment the peptide is selected from the group consisting of T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ ID NO: 4), N34(L6)C28 (SEQ ID NO: 5), and T-1249 (SEQ ID NO:6). In one embodiment, the peptide is T-20 (SEQ ID NO: 1).

As used herein, "T-20" and "DP178" are used interchangeably to denote a peptide having the following amino acid sequence: YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (SEQ ID NO:1) and as described [29,32]. DP107 has the following amino acid sequence: NNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQ (SEQ ID NO:2). N34 has the following amino acid sequence: SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQAR (SEQ ID NO:3). C28 has the following amino acid sequence: WMEWDREINNYTSLIHSLIEESQNQQEK (SEQ ID NO:4). N34(L6)C28 has

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the following amino acid sequence:
SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARSGGRGGWMEWDREINNYTSLIHS
LIEESQNQQEK (SEQ ID NO:5). T-1249 has the following amino
acid sequence : WQEWEQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF
(SEQ ID NO:6).

In one embodiment of the compositions described herein, the
peptide is a mutant peptide which (1)consists of amino
acids having a sequence identical to that of a wildtype
peptide selected from the group consisting of T-20 (SEQ ID
NO: 1), DP-107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ
ID NO: 4), N34(L6)C28 (SEQ ID NO: 5) and T-1249 (SEQ ID
NO:6), except for an addition of at least one glycine
residue to a 5' end of the peptide, to a 3' end of the
peptide, or to both ends of the peptide and (2) retards
gp41 from adopting a conformation capable of mediating
fusion of HIV-1 to a CD4+ cell by binding noncovalently to
an epitope on a gp41 fusion intermediate.

In one embodiment, the compound which retards gp41 from
adopting a conformation capable of mediating fusion of HIV-
1 to a CD4+ cell by binding noncovalently to an epitope on
a gp41 fusion intermediate is a fusion protein which
comprises a peptide selected from the group consisting of
T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO:
3), C28 (SEQ ID NO: 4), N34(L6)C28 (SEQ ID NO: 5), and T-
1249 (SEQ ID NO:6).

In one embodiment of the compositions described herein, the

peptide is selected from the group consisting of T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ ID NO: 4), N34(L6)C28 (SEQ ID NO: 5), and T-1249 (SEQ ID NO:6).

In one embodiment of the compositions described herein, the peptide is T-20 (SEQ-ID NO: 1). In one embodiment of the compositions described herein, the peptide is T-1249 (SEQ ID NO:6)

In one embodiment of the composition of this invention, the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a non-peptidyl agent.

In one embodiment, a non-peptidyl agent of the subject invention is a small molecule. In one embodiment, the non-peptidyl agent is a compound having a molecular weight less than 500 daltons.

In one embodiment of the composition of this invention, the antibody which binds to a CCR5 receptor includes but is not limited to PA8 (ATCC Accession No. HB-12605), PA10 (ATCC Accession No.12607), PA11 (ATCC Accession No. HB-12608), PA12 (ATCC Accession No. HB-12609), and PA14 (ATCC Accession No. HB-12610). In one embodiment, the antibody is a monoclonal antibody. In one embodiment, the monoclonal antibody is a human, humanized or chimeric antibody. In one

embodiment, the portion of the antibody is a Fab fragment of the antibody. In one embodiment, the portion of the antibody comprises the variable domain of the antibody. In one embodiment, the portion of the antibody comprises a CDR portion of the antibody. In one embodiment, the monoclonal antibody is an IgG, IgM, IgD, IgA, or IgE monoclonal antibody.

In one embodiment of the composition of this invention, the relative mass ratio of each such compound in the admixture ranges from about 25:1 to about 1:1. In one embodiment, the mass ratio is about 25:1. In one embodiment, the mass ratio is about 5:1. In one embodiment, the mass ratio is about 1:1.

In one embodiment of the composition of this invention, the composition is admixed with a carrier. The carriers of the subject invention include but are not limited to aerosol, intravenous, oral or topical carriers. Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may include but are not limited to aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's

dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with an amount of the composition of the subject invention effective to inhibit HIV-1 infection of the CD4+ cell so as to thereby inhibit HIV-1 infection of the CD4+ cell.

In one embodiment, the CD4+ cell is present in a subject and the contacting is effected by administering the composition to the subject.

As used herein, "subject" includes any animal or artificially modified animal capable of becoming HIV-infected. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The animals include but are not limited to mice, rats, dogs, cats, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human.

As used herein, "administering" may be effected or performed using any of the methods known to one skilled in

the art, which includes intralesional, intraperitoneal, intramuscular, subcutaneous, intravenous, liposome mediated delivery, transmucosal, intestinal, topical, nasal, oral, anal, ocular or otic delivery. The compounds may be administered separately (e.g., by different routes of administration, sites of injection, or dosing schedules) so as to combine in synergistically effective amounts in the subject.

The dose of the composition of the invention will vary depending on the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000 μg/kg. Based upon the composition, the dose can be delivered continuously, such as by continuous pump, or at periodic intervals. For example, on one or more separate occasions. Desired time intervals of multiple doses of a particular composition can be determined without undue experimentation by one skilled in the art.

As used herein, "effective dose" means an amount in sufficient quantities to either treat the subject or prevent the subject from becoming infected with HIV-1. A person of ordinary skill in the art can perform simple titration experiments to determine what amount is required to treat the subject.

In one embodiment, the effective amount of the composition comprises from about 0.000001 mg/kg body weight to about 100 mg/kg body weight of the subject.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (1) an amount of an antibody which binds to a CCR5 receptor and (2) an amount of a compound which

5 retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

10 This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (1) an amount of an antibody which binds to a CCR5 receptor, (2) an amount of a compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding

15 of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell effective to inhibit HIV-1 infection of the CD4+ cell, and (3) an amount of a compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding

20 noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

This invention provides a method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the

25 CD4+ cell with (1) an amount of a compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell effective to inhibit HIV-1 infection of the CD4+ cell and (2) an amount of a compound which retards

gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate so as to thereby inhibit HIV-1 infection of the CD4+ cell.

In one embodiment, the CD4+ cell is present in a subject and the contacting is effected by administering the compounds to the subject. In one embodiment, the compounds are administered to the subject simultaneously. In one embodiment, the compounds are administered to the subject at different times. In one embodiment, the compounds are administered to the subject by different routes of administration.

The subject invention has various applications which includes HIV treatment such as treating a subject who has become afflicted with HIV. As used herein, "afflicted with HIV-1" means that the subject has at least one cell which has been infected by HIV-1. As used herein, "treating" means either slowing, stopping or reversing the progression of an HIV-1 disorder. In the preferred embodiment, "treating" means reversing the progression to the point of eliminating the disorder. As used herein, "treating" also means the reduction of the number of viral infections, reduction of the number of infectious viral particles, reduction of the number of virally infected cells, or the amelioration of symptoms associated with HIV-1. Another application of the subject invention is to prevent a subject from contracting HIV. As used herein, "contracting

HIV-1" means becoming infected with HIV-1, whose genetic information replicates in and/or incorporates into the host cells. Another application of the subject invention is to treat a subject who has become infected with HIV-1. As used

5 herein, "HIV-1 infection" means the introduction of HIV-1 genetic information into a target cell, such as by fusion of the target cell membrane with HIV-1 or an HIV-1 envelope glycoprotein⁺ cell. The target cell may be a bodily cell of a subject. In the preferred embodiment, the target cell is

10 a bodily cell from a human subject. Another application of the subject invention is to inhibit HIV-1 infection. As used herein, "inhibiting HIV-1 infection" means reducing the amount of HIV-1 genetic information introduced into a target cell population as compared to the amount that would

15 be introduced without said composition.

This invention will be better understood from the Experimental Details that follow. However, one skilled in the art will readily appreciate that the specific methods

20 and results discussed are merely illustrative of the invention as described more fully in the claims that follow thereafter.

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## Experimental Details

### FIRST SERIES OF EXPERIMENTS
A. Materials and Methods

1) Reagents

Purified recombinant CD4-IgG2 protein was produced by Progenics Pharmaceuticals, Inc. from plasmids CD4-IgG2-HC-pRcCMV and CD4-kLC-pRcCMV (ATCC Accession Nos. 75193 and 75194, respectively) as described [5]. HeLa-env cells were prepared by transfecting HeLa cells (ATCC Catalog # CCL-2) with HIV-1 gp120/gp41 env-expressing plasmid pMA243 as described [51]. PM1 cells are available from the National Institutes of Health AIDS Reagent Program (Catalog #3038). The T-20 peptide was synthesized using standard solid-phase Fmoc chemistry and purified and characterized as described [31,32].

2) Inhibition of HIV-1 env-mediated membrane fusion

HIV-1 envelope-mediated fusion between HeLa-Env$_{JR-FL}$ and PM1 cells was detected using a Resonance Energy Transfer (RET) assay. Equal numbers (2x104) of fluorescein octadecyl ester (F18)-labeled envelope-expressing cells and octadecyl rhodamine (R18)-labeled PM1 cells were plated in 96-well plates in 15% fetal calf serum in phosphate buffered saline and incubated for 4h at 37 (C in the presence of varying concentrations of CD4-IgG2, T-20 or combinations thereof. Fluorescence RET was measured with a Cytofluor plate-reader (PerSeptive Biosystems) and % RET was determined as

previously described [19].

3) Quantitative analysis of the synergistic, additive or antagonistic effects of combining the agents

HIV-1 inhibition data were analyzed according to the Combination Index method of Chou and Talay [52,53]. The data are modeled according to the median-effect principle, which can be written

$$f = 1/[1 + (K/c)^m]$$

(1)

where f is the fraction affected/inhibited, c is concentration, K is the concentration of agent required to produce the median effect, and m is an empirical coefficient describing the shape of the dose-response curve. Equation (1) is a generalized form of the equations describing Michaelis-Menton enzyme kinetics, Langmuir adsorption isotherms, and Henderson-Hasselbalch ionization equilibria, for which m = 1 in all cases. In the present case, K is equal to the $IC_{50}$ value. K and m are determined by curve-fitting the dose-response curves.

After the best-fit parameters for K and m are obtained for the experimental agents and their combination, Equation (1) is rearranged to allow for calculation of c for a given f. The resulting table of values (e.g., Figure X) is used to calculate the Combination Index (CI) using the equation

$$CI = c_{1m}/c_1 + c_{2m}/c_2 + c_{1m}c_{2m}/c_1c_2$$
(2)

*where*

$c_1$ = concentration of compound 1 when used alone

$c_2$ = concentration of compound 2 when used alone

$c_{1m}$ = concentration of compound 1 in the mixture

$c_{2m}$ = concentration of compound 2 in the mixture

All concentrations are those required to achieve a given degree of inhibition. Equation (2) is used when the molecules are mutually nonexclusive, i.e., have different sites of action. Since this is the likely scenario for inhibitors of HIV-1 attachment and gp41 fusion intermediates, Equation (2) was used for all Combination Index calculations. Mutually nonexclusive calculations provide a more conservative estimate of the degree of synergy that mutually exclusive calculations, for which the $c_{1m}c_{2m}/c_1c_2$ term is dropped. CI < 1 indicates synergy, CI = 1 indicates purely additive effects, and CI > 1 indicates antagonism. In general, CI values are most relevant at the higher levels of inhibition that are required to achieve a measurable clinical benefit.

B. Results and Discussion

Combinations of inhibitors of HIV-1 attachment and gp41 fusion intermediates were first tested for the ability to inhibit HIV-1 env-mediated membrane fusion in the RET

assay.  This assay has proven to be a highly successful model of the HIV-1 entry process.  In this assay, env-dependent coreceptor usage patterns and cellular tropisms of the parental viruses are accurately reproduced [19].  Indeed, the assay was instrumental in demonstrating that CCR5 functions as a requisite fusion coreceptor and acts at the level of viral entry [54].  The fusion assay and infectious virus are similarly sensitive to inhibition by metal chelators and agents that target the full complement of viral and cellular receptors [19,46,55].

Dose-response curves were obtained for the agents used individually and in combination in both assays. Data were analyzed using the median effect principle [52,53]. The concentrations of single-agents or their mixtures required to produce a given effect were quantitatively compared in a term known as the Combination Index (CI).  CI > 1 indicates antagonism, CI = 1 indicates a purely additive effect, and CI < 1 indicates a synergistic effect wherein the presence of one agent enhances the effect of another.

Combinations of CD4-IgG2 and T-20 were observed to be potently synergistic in inhibiting env-mediated membrane fusion.  Figure 1 illustrates representative dose-response curves obtained in the membrane fusion assay for CD4-IgG2, T-20, and combinations of the two. The curve for the combination is highly displaced towards lower inhibitor concentrations and provides qualitative evidence that CD4-IgG2 and T-20 act in a synergistic manner.

To quantitatively calculate the degree of synergy observed between CD4-IgG2 and T-20, we analyzed the dose-response curves according to the Combination Index method [52,53]. The analysis included data obtained at 25:1, 5:1, and 1:1

5    CD4-IgG2:T-20 mass ratios. At the 25:1 mass ratio, both high (0-250 $\mu$g/ml CD4-IgG2 and 0-10 $\mu$g/ml T-20) and low (0-50 $\mu$g/ml CD4-IgG2 and 0-2 $\mu$g/ml T-20) concentration ranges were evaluated. As indicated in Figure 2, potent synergies were observed over these broad ranges of inhibitor ratios

10   and concentrations, with CI values as low as 0.20 under optimal conditions. This degree of synergy is remarkable since CI values of 0.2 are rarely observed for combinations involving anti-HIV-1 antibodies [41-44], reverse transcriptase inhibitors [56], or protease inhibitors [57].

15   The observed synergies indicate that HIV-1 attachment and formation of gp41 fusion intermediates are inter-dependent steps. One possibility is that attachment inhibitors, when used at suboptimal concentrations, may slow but not abrogate the binding of gp120 to CD4. In this case, gp41

20   fusion intermediates may be formed and persist on the virus (or infected cell) for longer periods of time at levels below that required for membrane fusion and thus provide better targets for inhibitory agents.

25   The observed synergies translate into significant reductions in the amounts of CD4-IgG2 and T-20 needed for inhibition. These reductions are illustrated in Figure 3 for CD4-IgG2 and T-20 used in a 25:1 mass ratio. By way of example, inhibition of viral entry by 95% requires 0.21

$\mu$g/ml of T-20 used alone, 19 $\mu$g/ml of CD4-IgG2 used alone and 1.14 $\mu$g/ml of a combination containing 0.044 $\mu$g/ml of T-20 and 1.1 $\mu$g/ml of CD4-IgG2. The combination reduces the respective doses of T-20 and CD4-IgG2 by 5- and 17-fold, respectively. Still greater dose reductions are observed at higher levels of inhibition.

## Second Series of Experiments

HIV-1 entry proceeds via a cascade of at least three sequential events: (1) the attachment of the HIV-1 surface glycoprotein gp120 to CD4, which is the primary cellular receptor for HIV-1, (2) the interaction of the gp120-CD4 complex with fusogenic coreceptors such as CCR5 and CXCR4, and (3) membrane fusion mediated by the HIV-1 transmembrane glycoprotein gp41. PRO 542 (CD4-IgG2) is an antibody-like molecule that binds to gp120 and thereby inhibits attachment of the virus to host cells via CD4. PRO 140 (PA14) and PA12 are monoclonal antibodies to CCR5 that block its function as an HIV-1 coreceptor. Lastly, T-20 is a 36-mer peptide derived from the highly conserved C-terminal ectodomain of gp41. T-20 blocks gp41-mediated membrane fusion events. PRO 542 is thus an attachment inhibitor that blocks the first step of HIV-1 entry; PRO 140 and PA12 are both CCR5 coreceptor inhibitors that block the second step; and T-20 is a fusion inhibitor that blocks the third step. Attachment, coreceptor and fusion inhibitors are all members of a broad category of antiviral agents collectively know as HIV-1 entry inhibitors. CCR5 coreceptor inhibitors and CXCR4 coreceptor inhibitors

constitute two distinct subclasses of coreceptor inhibitors.

When used individually, each of these compounds inhibit HIV-1 infection in vitro. PRO 542 and T-20 have also both demonstrated significant antiviral activity when used individually in human clinical trials, providing clinical proof-of-concept for inhibitors of HIV-1 entry (1,2).

The multi-step, inter-dependent nature of HIV-1 entry suggests that combinations of entry inhibitors may act in a non-additive or cooperative manner that either enhances (synergizes) or diminishes (antagonizes) the antiviral effect. Significant synergies have been observed for certain 2-way combinations of entry inhibitors, including attachment inhibitors used with CCR5 coreceptor inhibitors, attachment inhibitors used with fusion inhibitors, CCR5 coreceptor inhibitors used with other CCR5 coreceptor inhibitors, and CXCR4 coreceptor inhibitors used with fusion inhibitors (3,4).

However, whereas synergies are observed with certain members of a given class of inhibitor, purely additive or even antagonistic effects are seen when other members of the same class are used (3), highlighting the complexity of the HIV-1 entry process and the difficulty of predicting synergistic combinations. No prior study has examined either 2-way combinations of CCR5 coreceptor inhibitors and fusion inhibitors or triple or higher combinations that

include members of all three classes of HIV-1 entry inhibitors. We have discovered that synergistic inhibition of HIV-1 can be obtained using the CCR5 coreceptor inhibitor PRO 140 in combination with the fusion inhibitor T-20. See Figure 4D. In addition, remarkable synergies are observed using a triple combination containing an attachment inhibitor (PRO 542), a CCR5 coreceptor inhibitor (either PRO 140 or PA12) and a fusion inhibitor (T-20). See Figures 4A-C and Figure 5. The synergies observed with the triple combination are surprisingly potent and translate into dose reductions ranging to 260-fold.

**REFERENCES FOR FIRST SERIES OF EXPERIMENTS**

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## SECOND SERIES OF EXPERIMENTS

Infection of cells by human immunodeficiency virus type 1
(HIV-1) is mediated by the viral envelope (env)
glycoproteins gp120 and gp41, which are expressed as a
noncovalent, oligomeric complex on the surface of virus and
virally infected cells. HIV-1 entry into target cells
proceeds at the cell surface through a cascade of events
that include (1) binding of the viral surface glycoprotein
gp120 to cell surface CD4, which is the primary receptor
for HIV-1, (2) env binding to fusion coreceptors such as
CCR5 and CXCR4, and (3) multiple conformational changes in
gp41. During fusion, gp41 adopts transient conformations
that include a prehairpin fusion intermediate that
ultimately folds into a conformation capable of mediating
fusion. These events culminate in fusion of the viral and
cellular membranes and the subsequent introduction of the
viral genome into the target cell. A similar sequence of
molecular events is required for infection to spread via
fusion of infected and uninfected cells. Each stage of the
viral entry process can be targeted for therapeutic
intervention.

HIV-1 attachment can be inhibited both by agents that bind
the viral envelope glycoproteins and by agents that bind
human CD4. Notably, HIV-1 attachment can be inhibited by
compounds that incorporate the gp120-binding domains of
human CD4 and molecular mimics thereof [1-7]. Because this
interaction between gp120 and CD4 is essential for virus

infection, CD4-based molecules have the potential to target most if not all strains of HIV-1. In addition, viruses have limited ability to develop resistance to such molecules.

The determinants for gp120 binding map to the first extracellular domain (D1) on CD4 [1], and the amino acids critical for binding center on a loop comprising amino acids 36-47. Potent HIV-1 inhibitory activity has been reproduced in a 27-amino acid peptide that mimics this loop and surrounding structures [7].

A number of recombinant CD4-based molecules have been developed and tested for clinical activity in man. The first of these contained the four extracellular domains (D1-D4) of CD4 but lacked the transmembrane and intracellular regions. This molecule, termed soluble CD4 (sCD4), demonstrated excellent tolerability when administered to humans at doses ranging to 10 mg/kg [8,9]. Transient reductions in plasma levels of infectious HIV-1 were observed in certain patients treated with sCD4. The short half-life of sCD4 in humans (45 minutes following intravenous administration) was identified as one obstacle to using this agent for chronic therapy.

Second-generation CD4-based proteins were developed with increased serum half-life. These CD4-immunoglobulin fusion proteins comprised the D1D2 domains of CD4 genetically fused to the hinge CH2 and CH3 regions of human IgG

molecules. These divalent proteins derive HIV-1 neutralizing capacity from their CD4 domains and Fc effector functions from the IgG molecule. A CD4-IgG1 fusion protein was shown to have excellent tolerability and improved pharmacokinetics in Phase I clinical testing [10]. The antiviral evaluations were inconclusive.

More recently, a third-generation tetravalent CD4-IgG2 fusion protein was developed that comprises the D1D2 domains of CD4 genetically fused to the heavy and light chain constant regions of human IgG2. This agent binds the HIV-1 envelope glycoprotein gp120 with nanomolar affinity [5] and may inhibit virus attachment both by receptor blockade and by detaching gp120 from the virion surface, thereby irreversibly inactivating the virus. In standard PBMC-based neutralization assays, CD4-IgG2 neutralized primary HIV-1 isolates derived from all major subtypes and outlier groups. The CD4-IgG2 concentrations required to achieve a 90% reduction in viral infectivity, the in vitro IC90, were approximately 15-20 $\mu$g/ml [11], concentrations that are readily achievable in vivo. CD4-IgG2 was similarly effective in neutralizing HIV-1 obtained directly from the plasma of seropositive donors in an ex vivo assay, indicating that this agent is active against the diverse viral quasispecies that are encountered clinically [12]. CD4-IgG2 also provided protection against infection by primary isolates in the hu-PBL-SCID mouse model of HIV-1 infection [13]. Recent analyses have demonstrated that CD4-IgG2's ability to neutralize primary viruses is independent

of their coreceptor usage [14].

Compared with mono- or divalent CD4-based proteins, CD4-IgG2 has consistently demonstrated as much as 100-fold greater potency at inhibiting primary HIV-1 isolates [5,12,14,15]. The heightened potency may derive from CD4-IgG2's ability to bind virions with increased valency/avidity and its steric juxtaposition of two gp120 binding sites on each Fab-like arm of the immunoglobulin molecule. The larger Fab-like arms of CD4-IgG2 are also more likely to span HIV-1 envelope spikes on the virion. In a variety of preclinical models, CD4-IgG2's anti-HIV-1 activity has been shown to compare favorably with those of the rare human monoclonal antibodies that broadly and potently neutralize primary HIV-1 isolates [5,11,14,15]. In addition, CD4-IgG2 therapy is in principle less susceptible to the development of drug-resistant viruses than therapies employing anti-env monoclonal antibodies or portions of the highly mutable HIV-1 envelope glycoproteins. These properties suggest that CD4-IgG2 may have clinical utility as an agent that neutralizes cell-free virus before it has the opportunity to establish new rounds of infection. In addition to treatment, CD4-IgG2 may have utility in preventing infection resulting from occupational, perinatal or other exposure to HIV-1.

In Phase I clinical testing, single-dose CD4-IgG2 demonstrated excellent pharmacology and tolerability. In addition, measurable antiviral activity was observed by

each of two measures. First, a statistically significant acute reduction in plasma HIV RNA was observed following administration of a single 10 mg/kg dose. In addition, sustained reductions in plasma levels of infectious HIV were observed in each of two patients tested. Taken together, these observations indicate that CD4-IgG2 possesses antiviral activity in humans [16].

In addition to CD4-based proteins and molecular mimics thereof, HIV-1 attachment can also be inhibited by antibodies and nonpeptidyl molecules. Known inhibitors include (1) anti-env antibodies such as IgG1b12 and F105 [17,18], (2) anti-CD4 antibodies such as OKT4A, Leu 3a, and humanized versions thereof [19,20], and (3) nonpeptidyl agents that target either gp120 or CD4 [21], [22-24]. The latter group of compounds includes aurintricarboxylic acids, polyhydroxycarboxylates, sulfonic acid polymers, and dextran sulfates.

Several agents have been identified that block HIV-1 infection by targeting gp41 fusion intermediates. These inhibitors may interact with the fusion intermediates and prevent them from folding into final fusogenic conformations. The first such agents to be identified comprised synthetic or recombinant peptides corresponding to portions of the gp41 ectodomain predicted to form hydrophobic alpha helices. One such region is present in both the amino and carboxy segments of the extracellular portion of gp41, and recent crystallographic evidence

suggests that these regions interact in the presumed fusogenic conformation of gp41 [25,26]. HIV-1 infection can be inhibited by agents that bind to either N- or C-terminal gp41 epitopes that are exposed during fusion. These agents
5       include the gp41-based peptides T-20 (formerly known as DP178), T-1249, DP107, N34, C28, and various fusion proteins and analogues thereof [27-33]. Other studies have identified inhibitors that comprise non-natural D-peptides and nonpeptidyl moieties [34,35]. Clinical proof-of-concept
10      for this class of inhibitors has been provided by T-20, which reduced plasma HIV RNA levels by as much as 2 logs in Phase I/II human clinical testing [36]. The broad antiviral activity demonstrated for this class of inhibitors reflects the high degree of gp41 sequence
15      conservation amongst diverse strains of HIV-1.

Recent studies [37] have demonstrated the possibility of raising antibodies against HIV-1 fusion intermediates. This work employed "fusion-competent" HIV vaccine
20      immunogens that capture transient fusion intermediates formed upon interaction of gp120/gp41 with CD4 and fusion coreceptors. The immunogens used in these studies were formalin-fixed cocultures of cells that express HIV-1 gp120/gp41 and cells that express human CD4 and CCR5 but
25      not CXCR4. The antibodies elicited by the vaccines demonstrated unprecedented breadth and potency in inhibiting primary HIV-1 isolates regardless of their coreceptor usage, indicating that the antibodies were raised against structures such as gp41 fusion intermediates

that are highly conserved and transiently exposed during HIV-1 entry. This class of antibodies does not include the anti-gp41 monoclonal antibody known as 2F5, which interacts with an epitope that is constitutively presented on virus particles prior to fusion [38].

Previously, synergistic inhibition of HIV-1 entry has been demonstrated using certain anti-env antibodies used in combination with other anti-env antibodies [39-44], anti-CD4 antibodies [45], or CD4-based proteins [6]. Similarly, synergies have been observed using anti-CCR5 antibodies used in combination with other anti-CCR5 antibodies, CC-chemokines, or CD4-based proteins [46]. However, no prior studies have examined the potential synergistic effects of combining inhibitors of gp41 fusion intermediates with inhibitors of other stages of HIV-1 entry. In particular, no studies have examined combinations of inhibitors of gp41 fusion intermediates and HIV-1 attachment.

A. Materials and Methods

1)    Reagents

Purified recombinant CD4-IgG2 protein was produced by Progenics Pharmaceuticals, Inc. from plasmids CD4-IgG2-HC-pRcCMV and CD4-kLC-pRcCMV (ATCC Accession Nos. 75193 and 75194, respectively) as described [5]. HeLa-env cells were prepared by transfecting HeLa cells (ATCC Catalog # CCL-2) with HIV-1 gp120/gp41 env-expressing plasmid pMA243 as described [51]. PM1 cells are available from the National

Institutes of Health AIDS Reagent Program (Catalog #3038).
The T-20 peptide was synthesized using standard solid-phase
*Fmoc* chemistry and purified and characterized as described
[31,32].

5

2)    Inhibition of HIV-1 env-mediated membrane fusion

HIV-1 envelope-mediated fusion between HeLa-Env$_{JR-FL}$ and PM1
cells was detected using a Resonance Energy Transfer (RET)
assay. Equal numbers (2x104) of fluorescein octadecyl ester

10    (F18)-labeled envelope-expressing cells and octadecyl
rhodamine (R18)-labeled PM1 cells were plated in 96-well
plates in 15% fetal calf serum in phosphate buffered saline
and incubated for 4h at 37 (C in the presence of varying
concentrations of CD4-IgG2, T-20 or combinations thereof.

15    Fluorescence RET was measured with a Cytofluor plate-reader
(PerSeptive Biosystems) and % RET was determined as
previously described [19].

3)    Quantitative analysis of the synergistic, additive or

20    antagonistic effects of combining the agents

HIV-1 inhibition data were analyzed according to the
Combination Index method of Chou and Talay [52,53]. The
data are modeled according to the median-effect principle,

25    which can be written

$$f \quad = \quad 1/[1 + (K/c)^m]$$

(1)

where $f$ is the fraction affected/inhibited, $c$ is concentration, K is the concentration of agent required to produce the median effect, and m is an empirical coefficient describing the shape of the dose-response curve. Equation (1) is a generalized form of the equations describing Michaelis-Menton enzyme kinetics, Langmuir adsorption isotherms, and Henderson-Hasselbalch ionization equilibria, for which m = 1 in all cases. In the present case, K is equal to the $IC_{50}$ value. K and m are determined by curve-fitting the dose-response curves.

After the best-fit parameters for K and m are obtained for the experimental agents and their combination, Equation (1) is rearranged to allow for calculation of $c$ for a given f. The resulting table of values (e.g., Figure X) is used to calculate the Combination Index (CI) using the equation

$$CI = c_{1m}/c_1 + c_{2m}/c_2 + c_{1m}c_{2m}/c_1c_2$$

(2)

where

$c_1$ = concentration of compound 1 when used alone

$c_2$ = concentration of compound 2 when used alone

$c_{1m}$ = concentration of compound 1 in the mixture

$c_{2m}$ = concentration of compound 2 in the mixture

All concentrations are those required to achieve a given degree of inhibition. Equation (2) is used when the molecules are mutually nonexclusive, i.e., have different sites of action. Since this is the likely scenario for inhibitors of HIV-1 attachment and gp41 fusion intermediates, Equation (2) was used for all Combination Index calculations. Mutually nonexclusive calculations provide a more conservative estimate of the degree of synergy that mutually exclusive calculations, for which the $c_{1m}c_{2m}/c_1c_2$ term is dropped. CI < 1 indicates synergy, CI = 1 indicates purely additive effects, and CI > 1 indicates antagonism. In general, CI values are most relevant at the higher levels of inhibition that are required to achieve a measurable clinical benefit.

B. Results and Discussion

Combinations of inhibitors of HIV-1 attachment and gp41 fusion intermediates were first tested for the ability to inhibit HIV-1 env-mediated membrane fusion in the RET assay. This assay has proven to be a highly successful model of the HIV-1 entry process. In this assay, env-dependent coreceptor usage patterns and cellular tropisms of the parental viruses are accurately reproduced [19]. Indeed, the assay was instrumental in demonstrating that CCR5 functions as a requisite fusion coreceptor and acts at the level of viral entry [54]. The fusion assay and infectious virus are similarly sensitive to inhibition by metal chelators and agents that target the full complement

of viral and cellular receptors [19,46,55].

Dose-response curves were obtained for the agents used individually and in combination in both assays. Data were analyzed using the median effect principle [52,53]. The concentrations of single-agents or their mixtures required to produce a given effect were quantitatively compared in a term known as the Combination Index (CI). CI > 1 indicates antagonism, CI = 1 indicates a purely additive effect, and CI < 1 indicates a synergistic effect wherein the presence of one agent enhances the effect of another.

Combinations of CD4-IgG2 and T-20 were observed to be potently synergistic in inhibiting env-mediated membrane fusion. Figure 6 illustrates representative dose-response curves obtained in the membrane fusion assay for CD4-IgG2, T-20, and combinations of the two. The curve for the combination is highly displaced towards lower inhibitor concentrations and provides qualitative evidence that CD4-IgG2 and T-20 act in a synergistic manner.

To quantitatively calculate the degree of synergy observed between CD4-IgG2 and T-20, we analyzed the dose-response curves according to the Combination Index method [52,53]. The analysis included data obtained at 25:1, 5:1, and 1:1 CD4-IgG2:T-20 mass ratios. At the 25:1 mass ratio, both high (0-250 $\mu$g/ml CD4-IgG2 and 0-10 $\mu$g/ml T-20) and low (0-50 $\mu$g/ml CD4-IgG2 and 0-2 $\mu$g/ml T-20) concentration ranges were evaluated. As indicated in Figure 7, potent synergies

were observed over these broad ranges of inhibitor ratios and concentrations, with CI values as low as 0.20 under optimal conditions. This degree of synergy is remarkable since CI values of 0.2 are rarely observed for combinations involving anti-HIV-1 antibodies [41-44], reverse transcriptase inhibitors [56], or protease inhibitors [57]. The observed synergies indicate that HIV-1 attachment and formation of gp41 fusion intermediates are inter-dependent steps. One possibility is that attachment inhibitors, when used at suboptimal concentrations, may slow but not abrogate the binding of gp120 to CD4. In this case, gp41 fusion intermediates may be formed and persist on the virus (or infected cell) for longer periods of time at levels below that required for membrane fusion and thus provide better targets for inhibitory agents.

The observed synergies translate into significant reductions in the amounts of CD4-IgG2 and T-20 needed for inhibition. These reductions are illustrated in Figure 8 for CD4-IgG2 and T-20 used in a 25:1 mass ratio. By way of example, inhibition of viral entry by 95% requires 0.21 $\mu$g/ml of T-20 used alone, 19 $\mu$g/ml of CD4-IgG2 used alone and 1.14 $\mu$g/ml of a combination containing 0.044 $\mu$g/ml of T-20 and 1.1 $\mu$g/ml of CD4-IgG2. The combination reduces the respective doses of T-20 and CD4-IgG2 by 5- and 17-fold, respectively. Still greater dose reductions are observed at higher levels of inhibition.

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**THIRD SERIES OF EXPERIMENTS**

HIV-1 entry proceeds via a cascade of events that afford promising targets for therapy. PRO 542 neutralizes HIV-1 by blocking its attachment to CD4, and T-20 blocks gp41-mediated fusion. Both drugs have shown promise in Phase I/II clinical trials. Here the drugs were tested individually and in combination in preclinical models of HIV-1 infection, and inhibition data were analyzed for cooperativity using the Combination Index method. Synergistic inhibition of virus-cell and cell-cell fusion was observed for phenotypically diverse viruses over a broad range of drug concentrations, often resulting in 10-fold or greater dose reductions in vitro. Additional mechanism-of-action studies probed the molecular basis of the synergies. The markedly enhanced activity observed for the PRO 542:T-20 combination indicates that the multi-step nature of HIV-1 entry leaves the virus particularly vulnerable to combinations of entry inhibitors. These findings provide a strong rationale for evaluating combinations of these agents for therapy in vivo.

Despite recent advances, present therapies for human immunodeficiency virus type 1 (HIV-1) infection are limited by their failure to eradicate HIV-1, the emergence of multi-drug resistant variants, and significant toxicities. Accordingly, there is an urgent need for new therapies that target additional stages of the HIV-1 replicative cycle, such as viral entry.

HIV-1 entry comprises three steps that provide promising targets for therapy: (1) the attachment of the HIV-1 surface glycoprotein gp120 to CD4, (2) the interaction of the gp120-CD4 complex with a coreceptor, and (3) membrane fusion mediated by the HIV-1 transmembrane glycoprotein gp41. Attachment, coreceptor, and fusion inhibitors are broadly referred to as entry inhibitors and are currently in clinical development [1-4].

The attachment inhibitor PRO 542 (CD4-IgG2) is a tetravalent CD4-immunoglobulin fusion protein that potently neutralizes primary HIV-1 isolates [5]. In Phase I/II testing, PRO 542 has demonstrated excellent tolerability and pharmacology and has reduced viral loads as measured by plasma HIV RNA, plasma viremia, and cell-associated virus [1,2]. PRO 542 is currently in Phase II clinical testing.

The fusion inhibitor T-20 is a peptide derived from the C-terminal ectodomain of gp41. T-20 possesses broad-spectrum antiviral activity mediated by its ability to act upon transient gp41 fusion intermediates [6,7]. Although the precise structures of the fusion intermediates are not known, they are known as "pre-hairpin" intermediates based on the post-fusion conformation of gp41. T-20 mediated 1-2 $\log_{10}$ reductions in HIV RNA when used as a single agent in Phase I/II trials and has been associated with prolonged viral suppression when used with FDA-approved antiretrovirals [3,4]. T-20 has entered Phase III clinical

testing.

Combinations of antiretroviral medications are required for durable suppression of HIV-1 replication. PRO 542 and T-20 are attractive candidates for combination use because they are well-tolerated, "first-in-class" agents that target distinct stages of HIV-1 entry. Moreover, the multi-step, interdependent nature of HIV-1 entry suggests that these drugs might act in a non-additive manner. In this study, we evaluated these agents individually and in combination in vitro and analyzed the results for cooperative effects. The PRO 542:T-20 combination potently and synergistically inhibited entry of phenotypically diverse HIV-1 isolates over a broad range of experimental conditions. Additional studies shed light on the molecular basis for the enhanced antiviral activity.

MATERIALS AND METHODS

Compounds PRO 542 was expressed in Chinese hamster ovary cells and purified by column chromatography by Progenics [5]. T-20 was synthesized by solid-phase fluroenylmethoxycarbonyl chemistry, purified by reversed phase chromatography and analyzed for purity and size by HPLC and mass spectroscopy by Progenics. PRO 542 and T-20 were formulated in phosphate buffered saline and stored at -95°C.

HIV-1 virus-cell fusion assays $NLluc^+env^-$ viruses were complemented in trans by the envelope glycoproteins (Env)

from HIV-1$_{JR-FL}$ (R5) or HIV-1$_{DH123}$ (R5X4) [8]. HeLa-CD4$^+$CCR5$^+$ cells were infected with chimeric reporter viruses containing 50-100 ng/ml of p24 in the presence or absence of drugs. Cells were washed and fed fresh drug-containing media at 2 h and drug-free media at 12 h. After 72 h, cell lysates were assayed for luciferase activity [9].

**HIV-1 cell-cell fusion assays** Cell-cell fusion mediated by the envelope glycoproteins of HIV-1$_{JR-FL}$ and HIV-1$_{LAI}$ was measured using a fluorescence resonance energy transfer (RET) assay [10], which measures fusion between HeLa cells expressing HIV-1 envelope glycoproteins and CD4$^+$ target cells. The T cell lines PM1 and SupT1 were used as target cells in this study. PM1 endogenously express CCR5 and CXCR4. Cells are membrane-labeled with fluorescent dyes that possess overlapping excitation and emission spectra, such that RET occurs when the dyes are placed in the same membrane following fusion. The RET signal is directly related to the extent of membrane fusion.

**Quantitative analysis of drug interactions** Inhibition data from three independent assays were averaged and analyzed for cooperative effects using the Combination Index (CI) method [11]. In all analyses, PRO 542 and T-20 were assumed to act non-competitively, which leads to a more conservative estimate of synergy. CI values of <0.9 (or >1.1) were taken to represent meaningful synergy (or antagonism). Dose reductions were calculated as the ratio of drug concentrations required for inhibition when the

drug is used alone or in combination [11].


**RESULTS**

**Inhibition of HIV-1 virus-cell fusion** PRO 542, T-20 and 1:2

molar combinations thereof were used to inhibit HIV-1$_{JR-FL}$

and HIV-1$_{DH123}$ entry. For HIV-1$_{JR-FL}$ (Figure 9a), the

concentration required for 90% inhibition (IC$_{90}$) was 12 nM

for PRO 542, 45 nM for T-20 and 4.1 nM for the combination

(1.5 nM PRO 542 and 2.6 nM T-20). Favorable drug-drug

interactions are evident from the fact that the IC$_{90}$ of the

combination is substantially lower than that of either

compound alone.

Combination Index analyses (Figure 10) demonstrated that

potent PRO 542:T-20 synergy was observed for both HIV-1$_{JR-FL}$

and HIV-1$_{DH123}$. Over the range of 50-95% inhibition, CI

values ranged from 0.39-0.14 for HIV-1$_{JR-FL}$ and 1.1-0.36 for

HIV-1$_{DH123}$. Approximately 10-fold less PRO 542 and 20-fold

less T-20 was needed to inhibit HIV-1$_{JR-FL}$ entry by 90-95%.

Significant dose reductions were also observed for HIV-1$_{DH123}$

(Figure 10).


**Inhibition of HIV-1 Env-mediated cell-cell fusion** PRO 542,

T-20 and 1:10 molar combinations thereof were used to

inhibit HIV-1$_{JR-FL}$ Env-mediated cell-cell fusion in the RET

assay (Figure 9b). IC$_{90}$ values were 11 nM for PRO 542, 22 nM

for T-20 and 5.5 nM for the combination (0.55 nM PRO 542

and 4.9 nM T-20). CI values ranged from 0.34-0.27 over the

range of 50-95% inhibition (Figure 10). Robust synergies

were also observed using PRO 542:T-20 molar ratios of 1:2

and 1:50 and translated into dose reductions ranging to 50-fold (Figure 10).

PRO 542 and T-20 also synergistically inhibited HIV-1$_{LAI}$
(X4) Env-mediated fusion. At a 1:10 PRO 542:T-20 ratio, CI
values ranged from 0.45-0.28 at 50-95% inhibition. The
dose reductions ranged from 12-fold to 30-fold for PRO 542
and from 2.5-fold to 5.4-fold for T-20. Synergy was also
observed when the SupT1 T cell line was used instead of PM1
(data not shown).

Mechanistic studies of PRO 542:T-20 synergy T-20 does not
interact with Env in its pre-fusion conformation but rather
targets transient gp41 structures [7]. PRO 542, on the
other hand, neutralizes free virus and triggers Env
conformational changes that are required for entry [12].
PRO 542 could conceivably trigger formation of gp41 fusion
intermediates, enabling T-20 to act on free virus. To
evaluate this hypothesis, PRO 542 and T-20 were pre-
incubated alone and in combination for 1 h at 37 °C with
HeLa-Env cells prior to washing and the addition of PM1
cells (Figure 9c). Whereas PRO 542 demonstrated 6-fold
lower but nonetheless significant potency, T-20 was
essentially inactive in this setting. The potency of the
PRO 542:T-20 mixture mirrored that of its PRO 542 content,
indicating that T-20 was inactive in the mixture and
suggesting that PRO 542 does not trigger formation of the
pre-hairpin intermediate. This result also indicates that
PRO 542:T-20 synergy does not reflect direct binding

between the compounds. Neither drug demonstrated activity when pre-incubated with PM1 cells prior to washing (data not shown).

5    Further experiments explored the conditions required to form the pre-hairpin intermediate. HeLa-Env and PM1 cells were co-incubated for 2 h at 25 °C, a temperature that is not permissive for HIV-1 fusion [13]. Inhibitor was added, and the cells were warmed to 37 °C. Whereas PRO 542 was

10   measurably less potent in blocking fusion under these conditions, T-20 was fully active (Figure 1d). Thus at 25 °C, HIV-1 fusion can proceed past gp120-CD4 attachment but is arrested at or before the formation of the pre-hairpin intermediate. Similar results were obtained when the cells

15   were incubated 16 h at 25 °C prior to the addition of inhibitor (data not shown).

Lastly, HeLa-Env cells, PM1 cells, and inhibitor (PRO 542, T-20 or a 1:10 combination) were co-incubated for 2 h at 25

20   °C prior to washing and warming to 37 °C. Under these conditions, PRO 542 demonstrated ~12-fold lower but nonetheless significant activity; T-20 was essentially inactive (>250-fold increase in $IC_{50}$); and the combination possessed activity consistent with its PRO 542 content

25   (data not shown). Taken together, the results indicate that the pre-hairpin intermediate forms only when Env, CD4 and coreceptor interact at a fusion-permissive temperature.

DISCUSSION

HIV-1 entry is a promising target for a new generation of antiretroviral therapies, and PRO 542 and T-20 are "first-in-class" investigational agents that block distinct stages of this highly cooperative process. Recognizing that combinations of entry inhibitors may emerge as a new paradigm for HIV-1 therapy, this study explored PRO 542:T-20 combinations in two preclinical models of HIV-1 infection. Potent synergy was observed for both virus-cell and cell-cell fusion, for phenotypically diverse virus isolates, and over a broad range of drug concentrations. In many instances, the synergy translated into 10-fold or greater dose reductions for PRO 542 and T-20 in vitro. Thus the multi-step nature of HIV-1 entry appears to leave the virus particularly vulnerable to cooperative inhibition. The findings provide a strong rationale for combination clinical trials designed to maximize the therapeutic potential of each compound.

If borne out in vivo, these findings could translate into a number of important clinical benefits. First, more potent therapies reduce the emergence of drug-resistant virus and thereby preserve a patient's therapeutic options. In addition, by reducing the amount of PRO 542 and T-20 required for therapy, the synergy could enable simplified dosing regimens. Dose-sparing regimens are in general also toxicity-sparing, although this may be of less concern for PRO 542 and T-20, which have been well-tolerated in clinical testing to date.

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For virus-cell fusion, larger dose reductions were seen for T-20 than for PRO 542, whereas the reverse was true for cell-cell fusion. The relative dose reductions reflect the complementary mechanisms of action of the two products: PRO 542 but not T-20 neutralizes cell-free virus, and T-20 is particularly effective in blocking cell-cell fusion [6-8]. By exploiting the in vitro abilities of each agent to potently block the two major modes of HIV-1 transmission, PRO 542:T-20 combinations may prove to be particularly effective in vivo.

PRO 542 and T-20 synergistically inhibited fusion mediated by R5, X4 and R5X4 virus envelopes. While the activity of PRO 542 against primary viruses is independent of their coreceptor usage [8], T-20's ability to block virus-cell fusion reportedly varies according coreceptor usage [14]. Additional studies with a broader panel of viruses are needed to determine if PRO 542:T-20 synergy is subtly affected by coreceptor usage.

PRO 542 alone was not able to trigger formation of the gp41 pre-hairpin intermediate, which formed only when Env, CD4 and coreceptor interacted at a fusion-permissive temperature. Thus synergy may derive from subtler events, such as the clustering of multiple gp120/gp41 trimers to form the fusion pore [13]. At sub-neutralizing doses, PRO 542 may block a subset of Envs from interacting with target cells, while perhaps allowing other Envs to proceed to the

pre-hairpin intermediate. The fusion intermediates may remain exposed and susceptible to T-20 for significantly longer periods of time, as required to recruit a critical number of free Envs to the site of the fusion pore. In a recent study, T-20 mediated 100% inhibition when added within the first 15 minutes of cell mixing but was essentially inactive (<10% inhibition) at 75 minutes [15]. Ongoing studies are examining the effects of PRO 542 during this time period.

Additional studies are exploring higher combinations of attachment, coreceptor and fusion inhibitors. Also, the present study employed only subtype B viruses. It will be important to determine if the combination inhibits HIV-1 in a genetic subtype-independent manner, as do PRO 542 and T-20 individually. Lastly, further studies will explore combinations of PRO 542 and T-20 against replication-competent viruses in primary cells both in vitro and in animal models of HIV-1 infection.

In summary, PRO 542 and T-20 were potently synergistic when used to inhibit HIV-1 entry in vitro. Cooperative inhibition of virus-cell and cell-cell fusion was observed for phenotypically diverse viruses and translated into meaningful dose reductions. These findings provide a strong rationale for evaluating combinations of these agents in vivo.

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**Fourth Series of Experiments**

Mechanisms of synergy between HIV-1 Attachment, Coreceptor and Fusion Inhibitors

HIV-1 entry proceeds via a cascade of events that provide promising targets for a new generation of antiviral therapies, including the gp120-CD4 attachment inhibitor PRO542, the gp120-coreceptor inhibitor PRO140, and the gp41 fusion inhibitors T-20 and T-1249. The multi-step nature of HIV-1 entry leaves the virus highly susceptible to inhibition by combinations of drugs that act at distinct stages of this process. It has been shown that double and triple drug cocktails of attachment, coreceptor and the fusion inhibitors potently and synergistically block HIV-1 entry over a wide range of experimental conditions in vitro.

The present study was performed in order to identify the molecular basis of the observed synergistic interactions. HIV-1 membrane fusion was monitored in real time using a semi-automatic fluorometric assay, and the inhibitory activities of the individual drugs and drug cocktails were evaluated in time-of-addition, washout, and stepwise temperature-controlled studies. The data were consistent with a model wherein the drugs act cooperatively to delay the recruitment of a critical number of fusion-active HIV-1 envelope glycoproteins to the site of the fusion pore. These findings have important implications for the combination use of HIV-1 entry inhibitors in vivo, which

may emerge as an important new paradigm for antiviral therapy.

As shown in Figure 11, HIV-1 entry involves at least three steps that provide targets for therapy: (1) gp120-CD4 attachment; (2) gp120-coreceptor interactions; and (3) gp41-mediated fusion.

The CD4-IgG2 attachment inhibitor (PRO 542), as shown in Figure 12. Is a tetravalent CD4-based protein. It broadly and potently neutralizes primary HIV isolates in vitro, ex vivo and in vivo, and acts prior to fusion. PRO542 has completed two phase I/II clinical trials, showing (1) excellent safety and pharmacokinetic profiles and that it is non-immunogenic; (2) statistically significant reductions in viral load with a single dose; and (3) sustained reductions in free and cell-associated infectious virus. PRO 542 is in Phase II testing.

The PRO 140 coreceptor inhibitor, as shown in Figure 13, is a monoclonal antibody to CCR5. It potently neutralizes CCR5-using virus. It protects primary T cells and macrophages, and its inhibition is genetic subtype-independent. It selectively blocks HIV entry without affecting chemokine-induced signaling. There has been no emergence of HIV resistance following 31 weeks in vitro culture.

The T-20 fusion inhibitor, as shown in 14, is a peptide

derived from HIV-1 gp41. It broadly and potently blocks HIV-1 cell-cell and cell-virus fusion in vitro. It has completed Phase I/II clinical trials, showing (1) good tolerability; (2) pK supports twice daily subcutaneous dosing; and (3) clinically significant dose-dependent reductions in viral load.

## Combination Use of Entry Inhibitors

Combination therapy may be required to control HIV in vivo. HIV entry inhibitors may represent the next major advance in therapy. It is critical to determine drug-drug interactions earlier rather than later in clinical development to avoid antagonistic combinations and identify synergistic combinations. The clinical benefits of synergistic combinations include more potent antiviral effects and more durable antiviral effects.

An HIV-1 virus-cell fusion assay is described in Figure 15. In a single-cycle HIV-1 entry assay, Env-Luc+ HIV reporter viruses were prepared as described [1]. The viruses are complemented with gp120/gp41 from an HIV strain of interest and thus are capable of fusing with target cells that express CD4 and fusion coreceptors, resulting in the introduction of the viral genes into the cell. Because the viruses encode the luciferase reporter enzyme rather than HIVenv, infection leads to the production of luciferase rather than new viral particles. The amount of luciferase is measured 72 hours post-infection using standard methods.

Figures 16 and 17 demonstrate the synergistic inhibition of virus-cell fusion with PRO542 and T-20.

An HIV-1 cell-cell fusion assay is described in Figure 18. Such assay is a Resonance Energy Transfer (RET) assay of HIV-1 membrane fusion. Cells expressing gp120/gp41 (ENV cells) are labeled with the fluorescent dye octadecyl fluorescsin (F18), while T cell that express CD4 and fusion coreceptors (CD4 cells) are labeled with octadecyl rhodamine (R18). The emission spectrum of F18 overlaps with the excitation spectrum of R18, allowing fluorescence RET to occur when the dyes are placed in the same membrane following fusion. RET is measured by stimulating F18 at 450nm and measuring the R18 emission at 590nm. The RET signal is directly related to the amount of HIV-1 fusion [2].

Figures 19 and 20 demonstrate the synergistic inhibition of cell-cell fusion with PRO542 and T-20.

Figures 21 and 22 demonstrates that the PRO140, PRO542 and T-20 triple combination synergistically blocks HIV-1 entry.

Figure 23 demonstrates that PRO542 does not potentiate T-20 activity in the absence of coreceptor. PRO542, T-20 or a 1:2 mixture were pre-incubated with HeLa-ENVJR-FL cells for 2 hours at 37°C prior to washing, addition of PM1 target cells, and completion of the fusion assay.

Formation of the prehairpin intermediates may need CD4, coreceptor and 37°C, as demonstrated in Figures 24 and 25.

Figures 26 and 27 describe a possible mechanism of synergy, wherein PRO542 increases the half-life of the T-20 targets.

Figure 28 demonstrates synergistic inhibition of HIV-1 entry using PRO542, T-1249 and in a 1:10 molar combination in an env-medicated membrane fusion (RET) assay. Figure 29 demonstrates the combination indices and dose reductions for a 1:10 molar combination of PRO542 and T-1249.

References for Fourth Series of Experiments:

1. Dragic et al, Nature 381:667.

2. Litwin et al., J. Virol. 70:6437.

What is claimed:

1. A composition which comprises an admixture of two compounds, wherein: (a) one compound is an antibody or portion thereof which binds to a CCR5 receptor; and (b) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

2. A composition which comprises an admixture of three compounds, wherein: (a) one compound is an antibody or portion thereof which binds to a CCR5 receptor; (b) one compound retards attachment of HIV-1 to a CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell; and (c) one compound retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate; wherein the relative mass ratio of any two of the compounds in the admixture ranges from about 100:1 to about 1:100, the composition being effective to inhibit HIV-1 infection of the CD4+ cell.

3. The composition of claim 2, wherein the compound which retards attachment of HIV-1 to the CD4+ cell by

retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a CD4-based protein.

5    4.    The composition of claim 3, wherein the CD4-based protein is a CD4-immunoglobulin fusion protein.

5.    The composition of claim 4, wherein the CD4-immunoglobulin fusion protein is CD4-IgG2, wherein the CD4-IgG2 comprises two heavy chains and two lights chains, wherein the heavy chains are encoded by an expression vector designated CD4-IgG2HC-pRcCMV (ATCC Accession No. 75193) and the light chains are encoded by an expression vector designated CD4-kLC-pRcCMV (ATCC Accession No. 75194).

6.    The composition of claim 2, wherein the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a protein, the amino acid sequence of which comprises that of a protein found in HIV-1 as an envelope glycoprotein.

7.    The composition of claim 6, wherein the protein binds to an epitope of CD4 on the surface of the CD4+ cell.

8.    The composition of claim 7, wherein the envelope glycoprotein is selected from the group consisting of

gp120, gp160, and gp140.

9.    The composition of claim 2, wherein the compound which retards the attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is an antibody or portion of an antibody.

10.   The composition of claim 9, wherein the antibody is a monoclonal antibody.

11.   The composition of claim 10, wherein the monoclonal antibody is a human, humanized or chimeric antibody.

12.   The composition of claim 9, wherein the portion of the antibody is a Fab fragment of the antibody.

13.   The composition of claim 9, wherein the portion of the antibody comprises the variable domain of the antibody.

14.   The composition of claim 9, wherein the portion of the antibody comprises a CDR portion of the antibody.

15.   The composition of claim 10, wherein the monoclonal antibody is an IgG, IgM, IgD, IgA, or IgE monoclonal antibody.

16.   The composition of claim 10, wherein the monoclonal

antibody binds to an HIV-1 envelope glycoprotein.

17. The composition of claim 16, wherein the HIV-1 envelope glycoprotein is selected from the group consisting of gp120 and gp160.

18. The composition of claim 16, wherein HIV-1 envelope glycoprotein is gp120 and the monoclonal antibody which binds to gp120 is IgG1b12 or F105.

19. The composition of claim 9, wherein the antibody binds to an epitope of CD4 on the surface of the CD4+ cell.

20. The composition of claim 2, wherein the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a peptide.

21. The composition of claim 2, wherein the compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell is a nonpeptidyl agent.

22. The composition of claim 1 or 2, wherein the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is an antibody.

23. The composition of claim 22, wherein the antibody is a monoclonal antibody.

24. The composition of claim 1 or 2, wherein the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a peptide.

25. The composition of claim 1 or 2, wherein the compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a fusion protein which comprises a peptide selected from the group consisting of T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ ID NO: 4), N34(L6)C28 (SEQ ID NO: 5), and T-1249 (SEQ ID NO:6).

26. The composition of claim 24, wherein the peptide is selected from the group consisting of T-20 (SEQ ID NO: 1), DP107 (SEQ ID NO: 2), N34 (SEQ ID NO: 3), C28 (SEQ ID NO: 4), N34(L6)C28 (SEQ ID NO: 5), and T-1249 (SEQ ID NO:6).

27. The composition of claim 24, wherein the peptide is T-20 (SEQ ID NO: 1).

28. The composition of claim 1 or 2, wherein the compound

which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate is a non-peptidyl agent.

29. The composition of claim 1 or 2, wherein the antibody which binds to a CCR5 receptor is selected from the group consisting of PA8 (ATCC Accession No. HB-12605), PA10 (ATCC Accession No.12607), PA11 (ATCC Accession No. HB-12608), PA12 (ATCC Accession No. HB-12609), and PA14 (ATCC Accession No. HB-12610).

30. The composition of claim 1 or 2, wherein the antibody is PA14 (ATCC Accession No. HB-12610).

31. The composition of claim 29, wherein the antibody is a monoclonal antibody.

32. The composition of claim 29, wherein the monoclonal antibody is a human, humanized or chimeric antibody.

33. The composition of claim 1 or 2, wherein the portion of the antibody is a Fab fragment of the antibody.

34. The composition of claim 1 or 2, wherein the portion of the antibody comprises the variable domain of the antibody.

35. The composition of claim 1 or 2, wherein the portion

of the antibody comprises a CDR portion of the antibody.

36.  The composition of claim 31, wherein the monoclonal antibody is an IgG, IgM, IgD, IgA, or IgE monoclonal antibody.

37.  The composition of claim 1 or 2, wherein the relative mass ratio of each such compound in the admixture ranges from about 25:1 to about 1:1.

38.  The composition of claim 37, wherein the mass ratio is about 25:1

39.  The composition of claim 37, wherein the mass ratio is about 5:1.

40.  The composition of claim 37, wherein the mass ratio is about 1:1.

41.  The composition of claim 1 or 2, wherein the composition is admixed with a carrier.

42.  The composition of claim 41, wherein the carrier is an aerosol, intravenous, oral or topical carrier.

43.  A method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with an amount of the composition of claim 1 or 2 effective to

inhibit HIV-1 infection of the CD4+ cell so as to thereby inhibit HIV-1 infection of the CD4+ cell.

44. The method of claim 43, wherein the CD4+ cell is present in a subject and the contacting is effected by administering the composition to the subject.

45. The method of claim 43, wherein the effective amount of the composition comprises from about 0.000001 mg/kg body weight to about 100 mg/kg body weight of the subject.

46. A method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (i) an amount of an antibody which binds to a CCR5 receptor and (2) an amount of a compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

47. A method of inhibiting HIV-1 infection of a CD4+ cell which comprises contacting the CD4+ cell with (1) an amount of an antibody which binds to a CCR5 receptor, (2) an amount of a compound which retards attachment of HIV-1 to the CD4+ cell by retarding binding of HIV-1 gp120 envelope glycoprotein to CD4 on the surface of the CD4+ cell effective to inhibit HIV-1 infection of

the CD4+ cell, and (3) an amount of a compound which retards gp41 from adopting a conformation capable of mediating fusion of HIV-1 to a CD4+ cell by binding noncovalently to an epitope on a gp41 fusion intermediate, so as to thereby inhibit HIV-1 infection of the CD4+ cell.

48. The method of claim 46 or 47, wherein the CD4+ cell is present in a subject and the contacting is effected by administering the compounds to the subject.

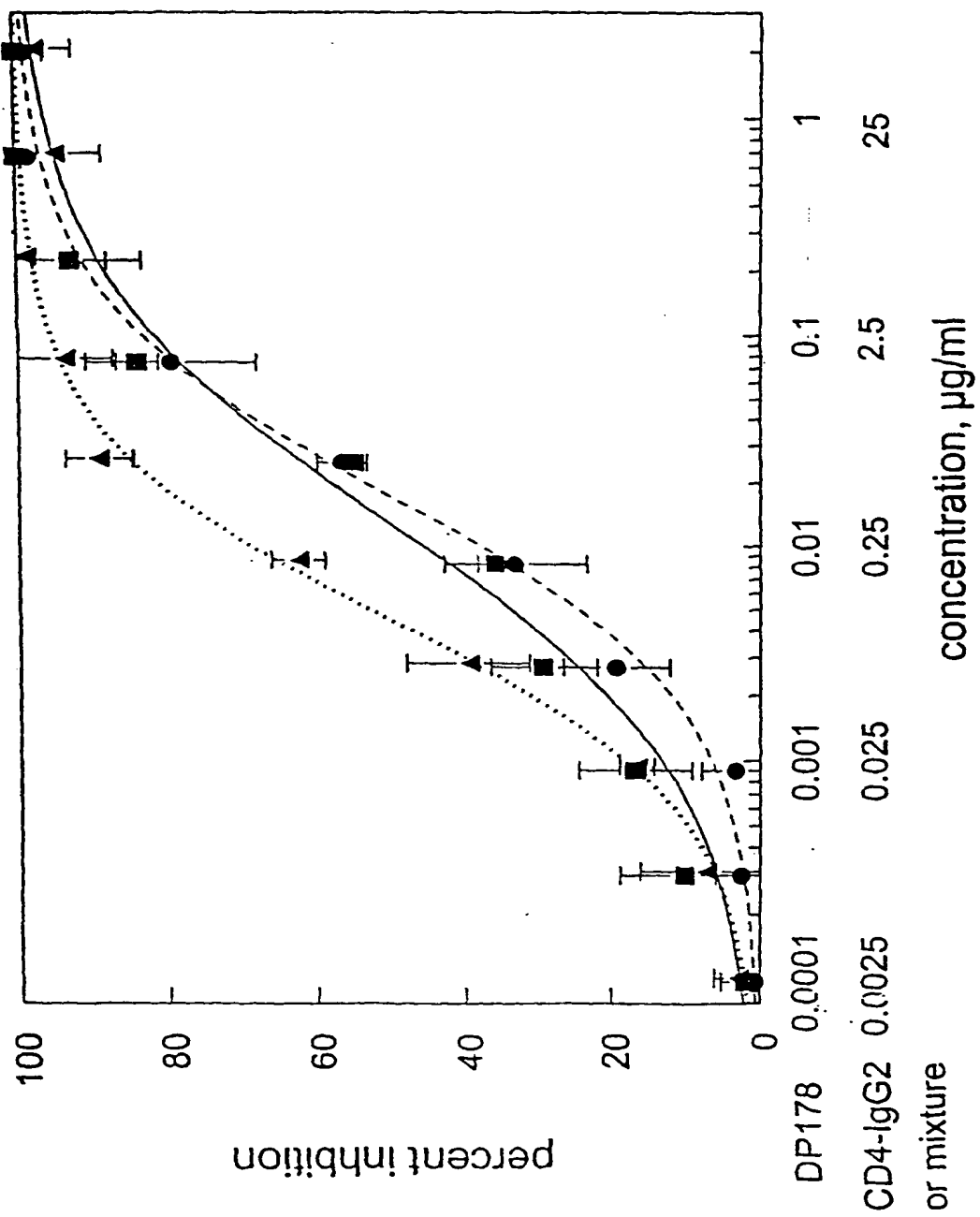49. The method of claim 48, wherein the compounds are administered to the subject simultaneously.

50. The method of claim 48, wherein the compounds are administered to the subject at different times.

51. The method of claim 48, wherein the compounds are administered to the subject by different routes of administration.

# Figure 1

Figure 2

| Percent Inhibition | Combination Index | | | |
|---|---|---|---|---|
| | CD4-IgG2:T-20 Mass Ratio | | | |
| | 25:1 (low) | 25:1 (high) | 5:1 | 1:1 |
| 95 | 0.32 | 0.20 | 0.22 | 0.50 |
| 90 | 0.38 | 0.25 | 0.27 | 0.55 |
| 85 | 0.43 | 0.29 | 0.30 | 0.59 |
| 80 | 0.47 | 0.33 | 0.34 | 0.62 |
| 75 | 0.51 | 0.36 | 0.37 | 0.65 |
| 70 | 0.54 | 0.39 | 0.40 | 0.67 |
| 65 | 0.58 | 0.42 | 0.43 | 0.70 |
| 60 | 0.61 | 0.45 | 0.45 | 0.73 |
| 55 | 0.65 | 0.48 | 0.49 | 0.75 |
| 50 | 0.69 | 0.51 | 0.52 | 0.78 |

# Figure 3

| Percent Inhibition | T-20 Concentration, μg/ml Alone | Combination | Dose Reduction | CD4-IgG2 Concentration, μg/ml Alone | Combination | Dose Reduction |
|---|---|---|---|---|---|---|
| 99 | 1.1 | 0.17 | 6.6 | 130 | 4.3 | 29 |
| 95 | 0.21 | 0.044 | 4.9 | 19 | 1.10 | 17 |
| 90 | 0.10 | 0.024 | 4.2 | 7.8 | 0.59 | 13 |
| 70 | 0.025 | 0.0076 | 3.3 | 1.6 | 0.19 | 8.4 |
| 50 | 0.011 | 0.0039 | 2.8 | 0.60 | 0.095 | 6.3 |

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# Figure 4A

| Percent Inhibition | Combination Index | PRO 542 Concentration, nM | | Dose Reduction | PA12 Concentration, nM | | Dose Reduction | T-20 Concentration, nM | | Dose Reduction |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Alone | Mix | | Alone | Mix | | Alone | Mix | |
| 95 | 0.41 | 10 | 2.1 | 4.8 | 730 | 2.8 | 260 | 94 | 19 | 4.9 |
| 90 | 0.45 | 7.0 | 1.6 | 4.4 | 320 | 2.1 | 150 | 63 | 14 | 4.5 |
| 70 | 0.47 | 4.1 | 0.92 | 4.5 | 72 | 1.2 | 60 | 30 | 8.1 | 3.7 |
| 50 | 0.48 | 3.1 | 0.66 | 4.7 | 28 | 0.87 | 32 | 19 | 5.8 | 3.3 |

PRO 542, PA12 and T-20 were used in an approximate 1:1:10 molar concentration ratio.

# Figure 4B

| Percent Inhibition | Combination Index | PRO 542 Concentration, nM | | | PRO 140 Concentration, nM | | | T-20 Concentration, nM | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Alone | Mix | Dose Reduction | Alone | Mix | Dose Reduction | Alone | Mix | Dose Reduction |
| 95 | 0.40 | 8.5 | 1.9 | 4.5 | 19 | 1.0 | 19 | 140 | 17 | 8.2 |
| 90 | 0.39 | 7.1 | 1.5 | 4.7 | 13 | 0.77 | 17 | 100 | 13 | 7.7 |
| 70 | 0.37 | 5.3 | 0.87 | 6.1 | 7.2 | 0.46 | 16 | 57 | 7.7 | 7.4 |
| 50 | 0.35 | 4.6 | 0.63 | 7.3 | 4.9 | 0.34 | 14 | 40 | 5.6 | 7.1 |

PRO 542, PRO 140 and T-20 were used in an approximate 2:1:20 molar concentration ratio.

# Figure 4C

| Percent Inhibition | Combination Index | PRO 542 Concentration, nM | | | PRO 140 Concentration, nM | | | T-20 Concentration, nM | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Alone | Mix | Dose Reduction | Alone | Mix | Dose Reduction | Alone | Mix | Dose Reduction |
| 95 | 0.24 | 61 | 2.5 | 24 | 11.9 | 0.72 | 17 | 156 | 22 | 7.1 |
| 90 | 0.22 | 32 | 1.4 | 23 | 8.4 | 0.40 | 21 | 96 | 13 | 7.4 |
| 70 | 0.19 | 9.8 | 0.50 | 20 | 4.5 | 0.14 | 32 | 40 | 4.5 | 8.9 |
| 50 | 0.18 | 4.7 | 0.26 | 18 | 3.0 | 0.074 | 41 | 23 | 2.3 | 10 |

PRO 542, PRO 140 and T-20 were used in an approximate 4:1:30 molar concentration ratio.

Figure 4D

| Percent Inhibition | Combination Index | PRO 140 | | | T-20 | | |
|---|---|---|---|---|---|---|---|
| | | Concentration, nM | | Dose Reduction | Concentration, nM | | Dose Reduction |
| | | Alone | Mix | | Alone | Mix | |
| 95 | 0.56 | 12 | 1.8 | 6.7 | 156 | 55 | 2.8 |
| 90 | 0.55 | 8.4 | 1.1 | 7.4 | 96 | 35 | 2.7 |
| 70 | 0.55 | 4.5 | 0.51 | 8.8 | 40 | 16 | 2.5 |
| 50 | 0.56 | 3.0 | 0.31 | 9.9 | 23 | 10 | 2.4 |

PRO 140 and T-20 were used in an approximate 1:30 molar concentration ratio.

Figure 5

*Triple Combination Synergistically Blocks HIV-1 Entry (I)*

Figure 6

Figure 7

## Combination Index

| Percent Inhibition | CD4-IgG2:T-20 Mass Ratio | | | |
| --- | --- | --- | --- | --- |
| | 25:1 (low) | 25:1 (high) | 5:1 | 1:1 |
| 95 | 0.32 | 0.20 | 0.22 | 0.50 |
| 90 | 0.38 | 0.25 | 0.27 | 0.55 |
| 85 | 0.43 | 0.29 | 0.30 | 0.59 |
| 80 | 0.47 | 0.33 | 0.34 | 0.62 |
| 75 | 0.51 | 0.36 | 0.37 | 0.65 |
| 70 | 0.54 | 0.39 | 0.40 | 0.67 |
| 65 | 0.58 | 0.42 | 0.43 | 0.70 |
| 60 | 0.61 | 0.45 | 0.45 | 0.73 |
| 55 | 0.65 | 0.48 | 0.49 | 0.75 |
| 50 | 0.69 | 0.51 | 0.52 | 0.78 |

# Figure 9

| Assay (virus) | PRO 542:T-20 Molar Ratio | Percent Inhibition | Combination Index | PRO 542 Concentration, nM | | Dose Reduction | T-20 Concentration, nM | | Dose Reduction |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Alone | Mix | | Alone | Mix | |
| Virus-cell fusion (JR-FL) | 1:2 | 95 | 0.14 | 30 | 2.8 | 11 | 120 | 5.1 | 24 |
| | | 90 | 0.18 | 12 | 1.5 | 8.0 | 45 | 2.6 | 17 |
| | | 70 | 0.29 | 2.5 | 0.44 | 5.7 | 8.0 | 0.78 | 10 |
| | | 50 | 0.39 | 0.92 | 0.21 | 4.4 | 2.7 | 0.37 | 7.3 |
| Virus-cell fusion (DH123) | 1:2 | 95 | 0.36 | 65 | 11 | 5.9 | 123 | 20 | 6.2 |
| | | 90 | 0.45 | 20 | 5.0 | 4.0 | 54 | 8.9 | 6.1 |
| | | 70 | 0.76 | 2.4 | 1.2 | 2.0 | 12 | 2.1 | 5.7 |
| | | 50 | 1.1 | 0.64 | 0.49 | 1.3 | 4.8 | 0.87 | 5.5 |
| Cell-cell fusion (JR-FL) | 1:2 | 95 | 0.36 | 35 | 6.3 | 5.6 | 73 | 11 | 6.6 |
| | | 90 | 0.43 | 14 | 3.2 | 4.4 | 34 | 5.8 | 5.9 |
| | | 70 | 0.61 | 2.9 | 0.94 | 3.1 | 8.5 | 1.7 | 5.0 |
| | | 50 | 0.76 | 1.0 | 0.43 | 2.3 | 3.6 | 0.78 | 4.6 |
| Cell-cell fusion (JR-FL) | 1:10 | 95 | 0.27 | 28 | 1.4 | 20 | 58 | 12 | 4.8 |
| | | 90 | 0.28 | 11 | 0.55 | 20 | 22 | 4.9 | 4.5 |
| | | 70 | 0.31 | 2.3 | 0.11 | 21 | 3.8 | 0.97 | 3.9 |
| | | 50 | 0.34 | 0.84 | 0.039 | 17 | 1.3 | 0.35 | 3.7 |
| Cell-cell fusion (JR-FL) | 1:50 | 95 | 0.33 | 47 | 0.84 | 56 | 120 | 37 | 3.2 |
| | | 90 | 0.34 | 15 | 0.30 | 50 | 42 | 13 | 3.2 |
| | | 70 | 0.36 | 1.8 | 0.045 | 40 | 6.1 | 2.0 | 3.0 |
| | | 50 | 0.38 | 0.49 | 0.014 | 35 | 1.8 | 0.61 | 3.0 |

Figure 8

| Percent Inhibition | T-20 Concentration, µg/ml | | Dose Reduction | CD4-IgG2 Concentration, µg/ml | | Dose Reduction |
|---|---|---|---|---|---|---|
| | Alone | Combination | | Alone | Combination | |
| 99 | 1.1 | 0.17 | 6.6 | 130 | 4.3 | 29 |
| 95 | 0.21 | 0.044 | 4.9 | 19 | 1.10 | 17 |
| 90 | 0.10 | 0.024 | 4.2 | 7.8 | 0.59 | 13 |
| 70 | 0.025 | 0.0076 | 3.3 | 1.6 | 0.19 | 8.4 |
| 50 | 0.011 | 0.0039 | 2.8 | 0.60 | 0.095 | 6.3 |

**Figure 10**

Figure 11

# HIV-1 Entry Involves at Least Three Steps that Provide Promising Targets for Therapy



gp120-CD4 attachment

gp120-coreceptor interactions

gp41-mediated fusion

Virus-Cell Fusion

Target Cell Membrane

Viral Membrane

CCR5

gp120

CD4

gp41
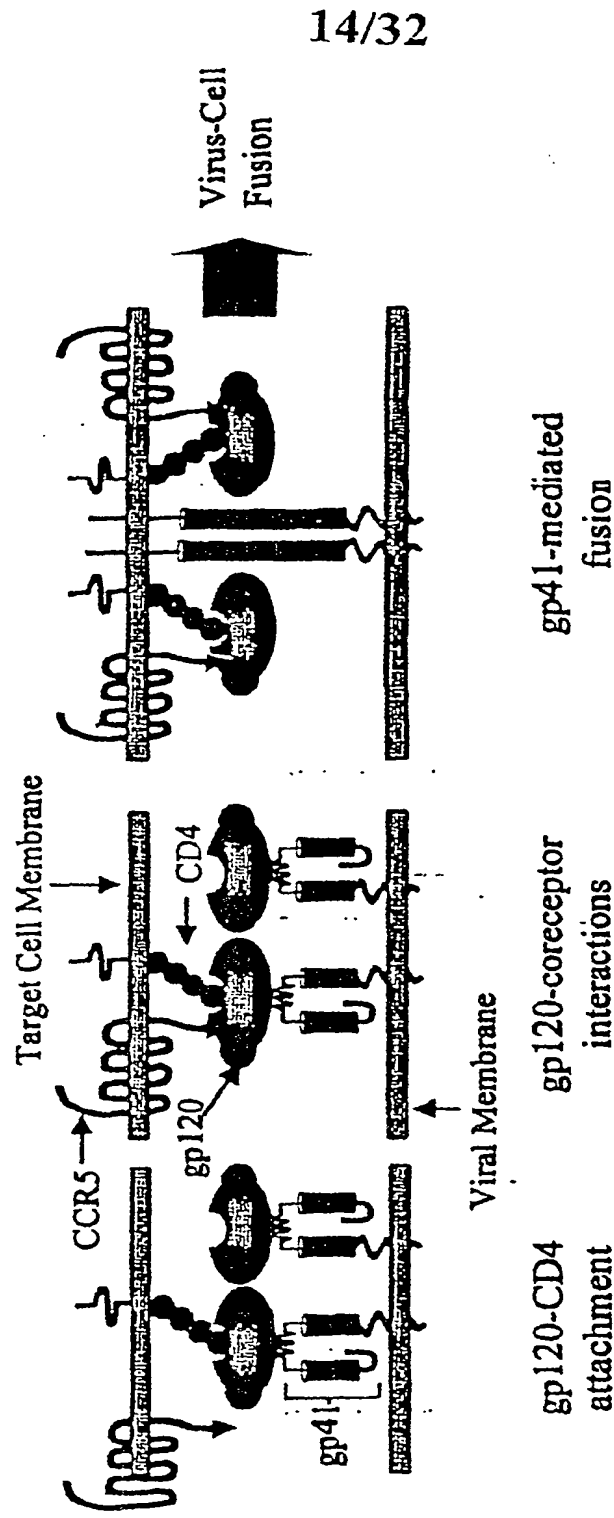
Figure 12

# *PRO 542 (CD4-IgG2)*
## *attachment inhibitor*
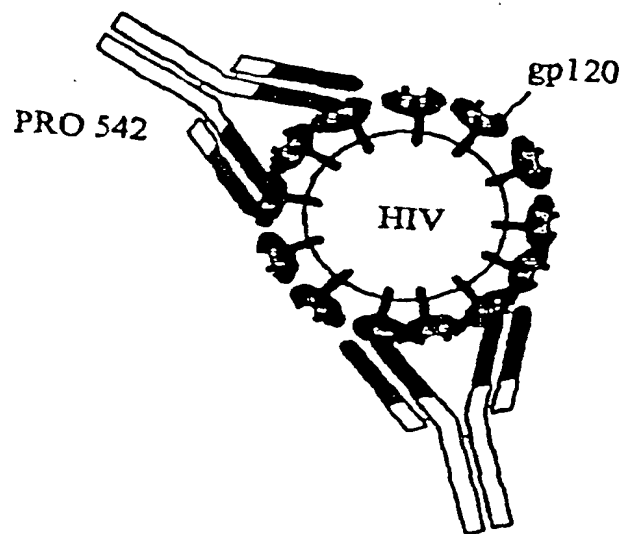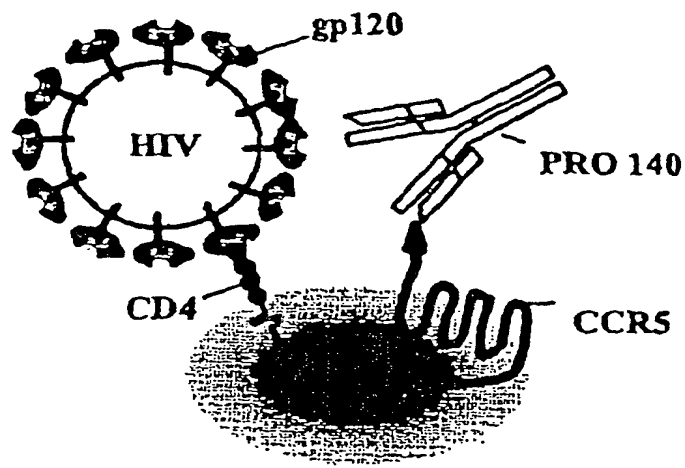
Figure 13

# PRO 140
## coreceptor inhibitor

Figure 14

# T-20
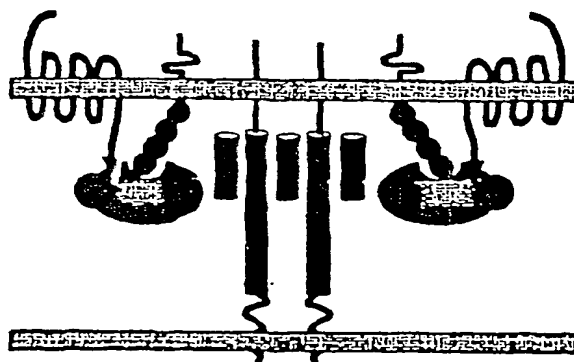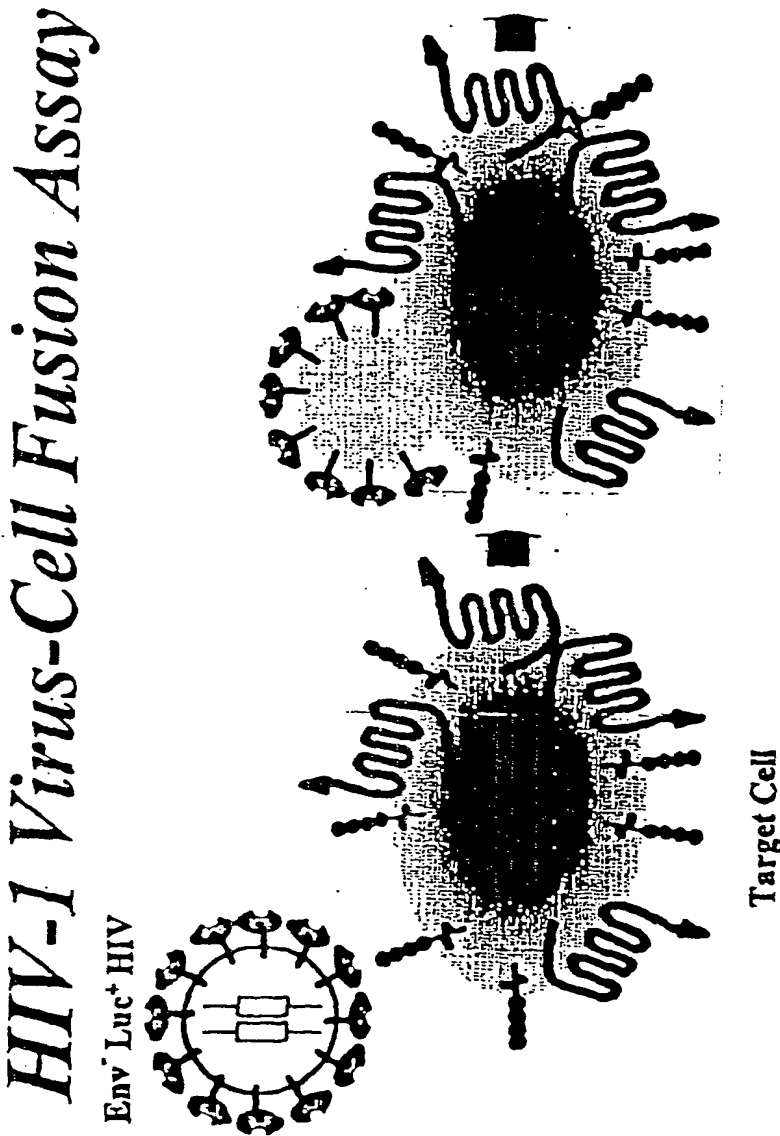## fusion inhibitor

Figure 15

# *HIV-1 Virus-Cell Fusion Assay*

Figure 16

# Synergistic Inhibition of Virus-Cell Fusion with PRO 542 and T-20 (I)
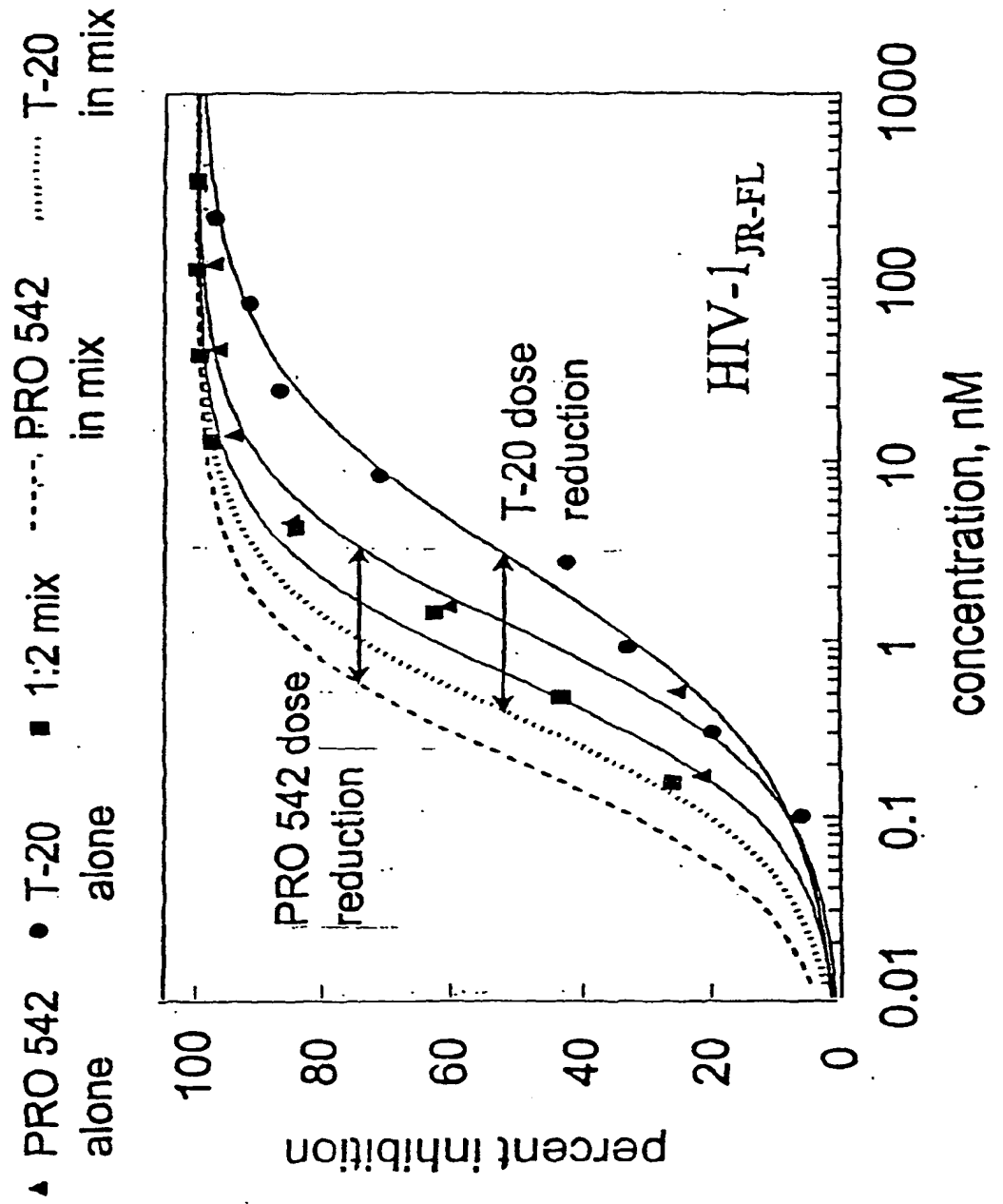
Figure 17

# Synergistic Inhibition of HIV-1 Virus-Cell-Fusion with PRO 542 and T-20 (II)

| | Percent Inhibition | Combination Index | Inhibitory Conc., nM | | Dose Reduction | |
|---|---|---|---|---|---|---|
| | | | PRO 542 | T-20 | PRO 542 | T-20 |
| JR-FL (R5) | 95 | 0.14 | 30 | 120 | 11 | 24 |
| | 90 | 0.18 | 12 | 45 | 8.0 | 17 |
| | 70 | 0.29 | 2.5 | 8.0 | 5.7 | 10 |
| | 50 | 0.39 | 0.92 | 2.7 | 4.4 | 7.3 |
| DH123 (R5X4) | 95 | 0.36 | 65 | 123 | 5.9 | 6.2 |
| | 90 | 0.45 | 20 | 54 | 4.0 | 6.1 |
| | 70 | 0.76 | 2.4 | 12 | 2.0 | 5.7 |
| | 50 | 1.1 | 0.64 | 4.8 | 1.3 | 5.5 |

PRO 542 and T-20 were used in a 1:2 molar ratio

Figure 18

# *HIV-1 Cell-Cell Fusion Assay*



450 nm

530 nm

ENV Cells

530 nm

590 nm
CD4 Cells

450 nm

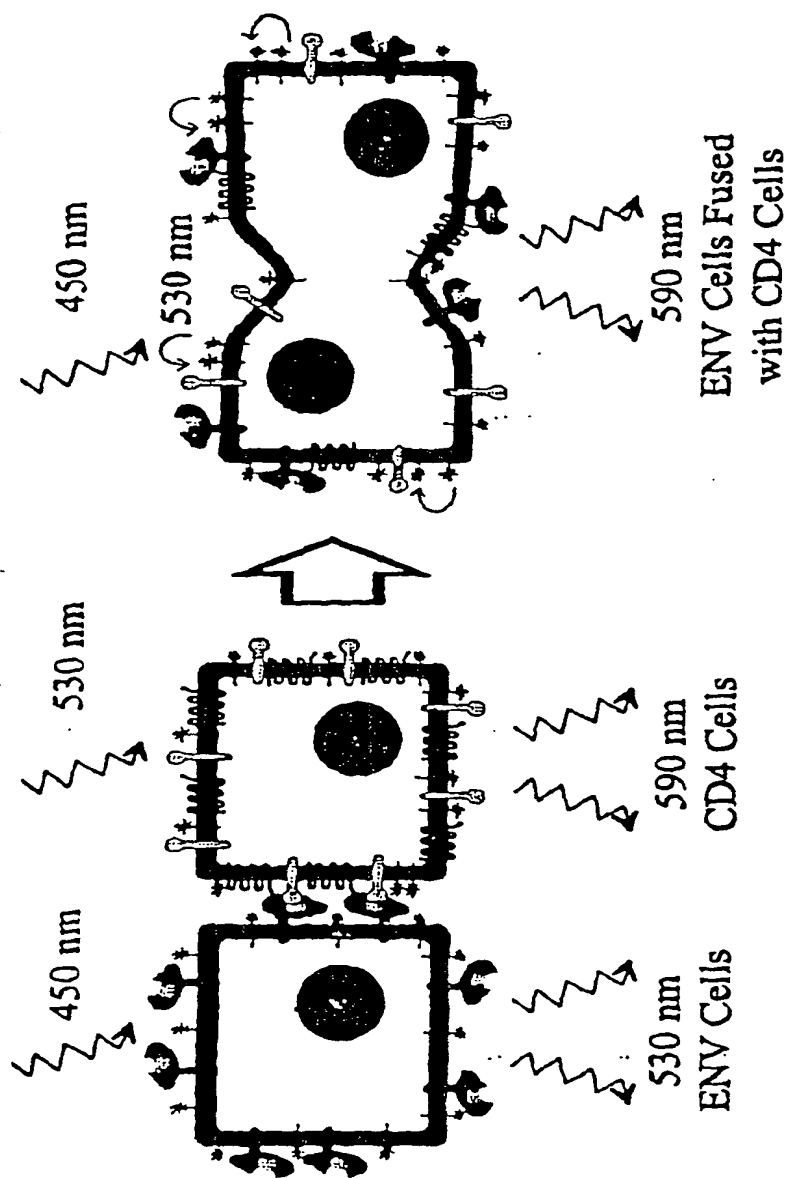530 nm

590 nm
ENV Cells Fused
with CD4 Cells

Figure 19

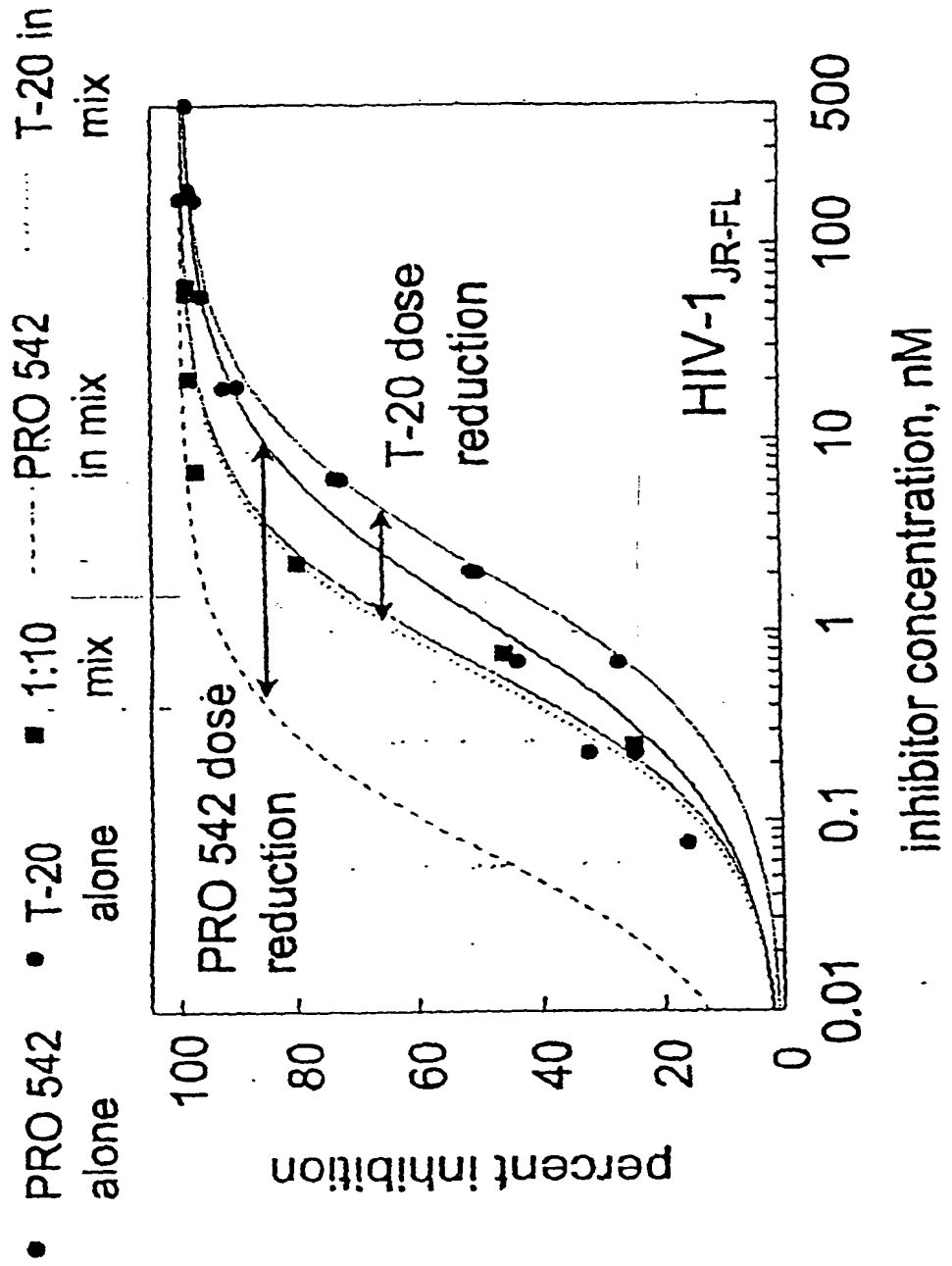# Synergistic Inhibition of Cell-Cell Fusion with PRO 542 and T-20 (I)

Figure 20

## Synergistic Inhibition of HIV-1 Cell-Cell Fusion with PRO 542 and T-20 (II)

| Conc. Ratio | Percent Inhibition | Combination Index | Inhibitory Conc, nM | | Dose Reduction (fold) | |
|---|---|---|---|---|---|---|
| | | | PRO 542 | T-20 | PRO 542 | T-20 |
| 1:2 | 95 | 0.32 | 95 | 47 | 17 | 4.9 |
| | 90 | 0.38 | 39 | 22 | 13 | 4.2 |
| | 50 | 0.69 | 3.0 | 2.5 | 6.2 | 2.8 |
| 1:10 | 95 | 0.27 | 28 | 58 | 20 | 4.8 |
| | 90 | 0.28 | 11 | 22 | 20 | 4.5 |
| | 50 | 0.34 | 0.84 | 1.3 | 22 | 3.7 |
| 1:50 | 95 | 0.33 | 47 | 120 | 56 | 3.2 |
| | 90 | 0.34 | 15 | 42 | 50 | 3.2 |
| | 50 | 0.38 | 0.49 | 1.8 | 35 | 3.0 |

Virus: HIV-1$_{JR-FL}$

Figure 21

## PRO 140, PRO 542 and T-20 Triple Combination Synergistically Blocks HIV-1 Entry (I)

- ◆ PRO 140 alone
- ▲ PRO 542 alone
- ● T-20 alone
- ■ 1:3:30 mix

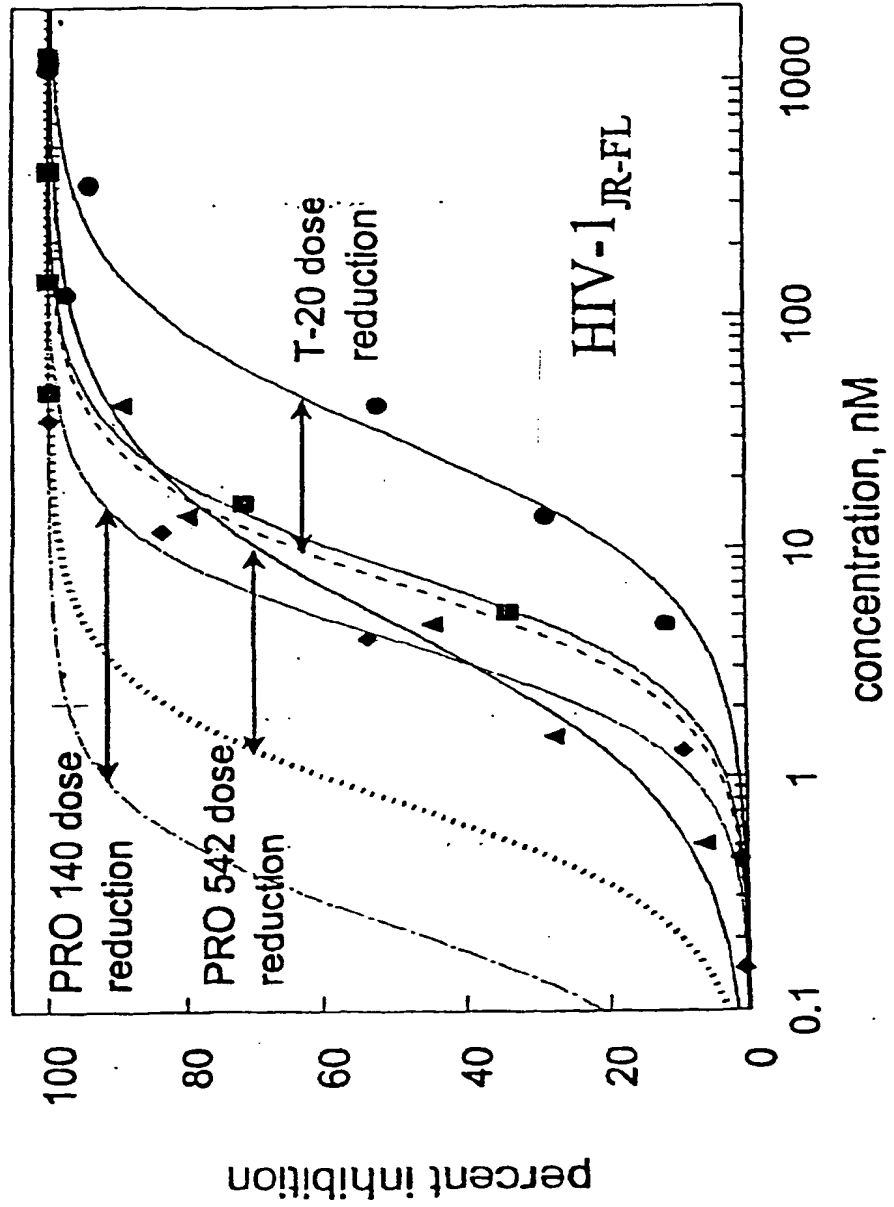—— PRO 140 in mix ······ PRO 542 in mix ----- T-20 in mix

Figure 22

## PRO 140, PRO 542, T-20 Triple Combination Synergistically Blocks HIV-1 Entry (II)

| Percent Inhibition | Combination Index | Inhibitory Conc, nM | | | Dose Reduction (fold) | | |
|---|---|---|---|---|---|---|---|
| | | PRO 140 | PRO 542 | T-20 | PRO 140 | PRO 542 | T-20 |
| 95 | 0.24 | 24 | 61 | 160 | 17 | 12 | 7.1 |
| 90 | 0.22 | 23 | 32 | 96 | 21 | 8.4 | 7.4 |
| 70 | 0.19 | 20 | 9.8 | 40 | 32 | 4.5 | 8.9 |
| 50 | 0.18 | 18 | 4.7 | 23 | 41 | 3.0 | 10 |

Inhibition of HIV-1$_{JR-FL}$ mediated cell-cell fusion with PRO 140, PRO 542 and T-20 used in a 1:3:30 molar ratio.

Figure 23

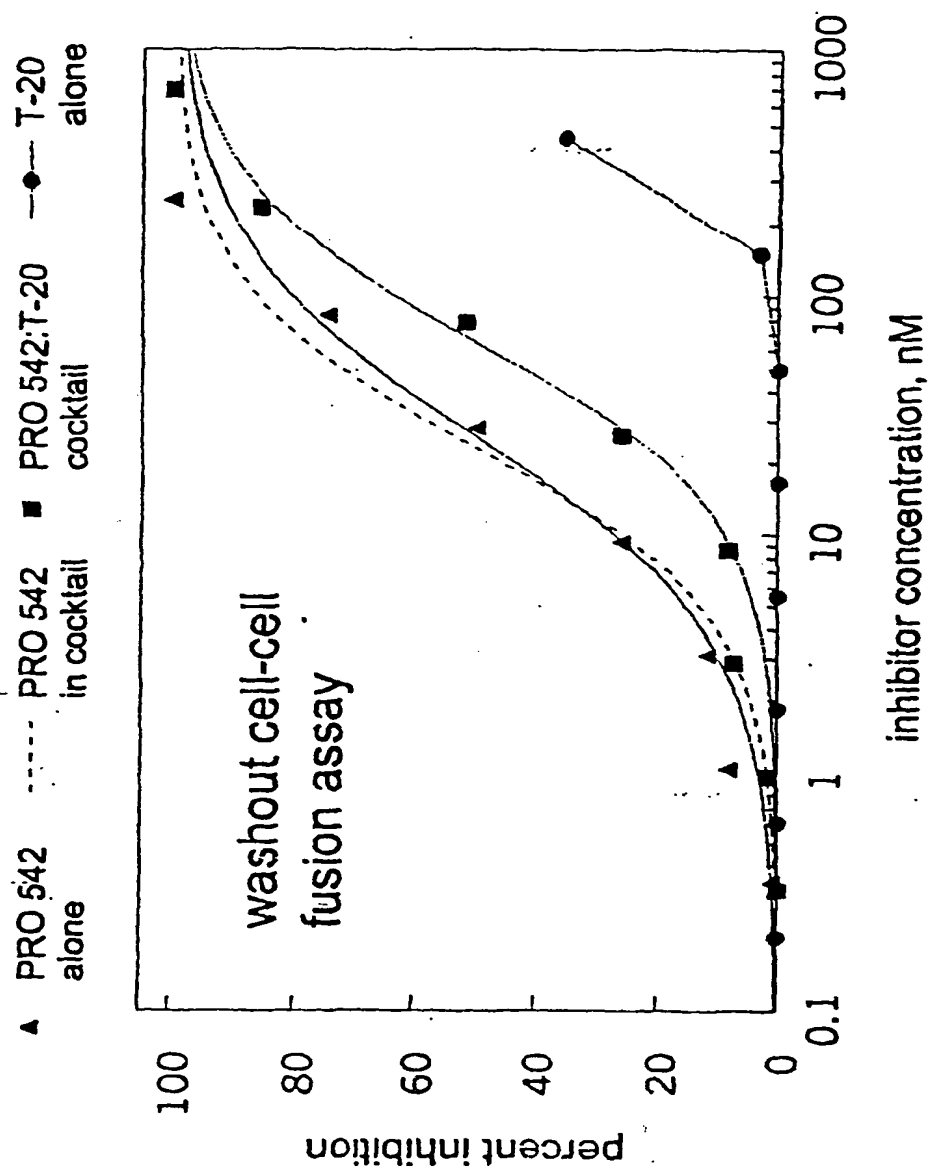*PRO 542 Does Not Potentiate T-20 Activity in the Absence of Coreceptor*

Figure 24

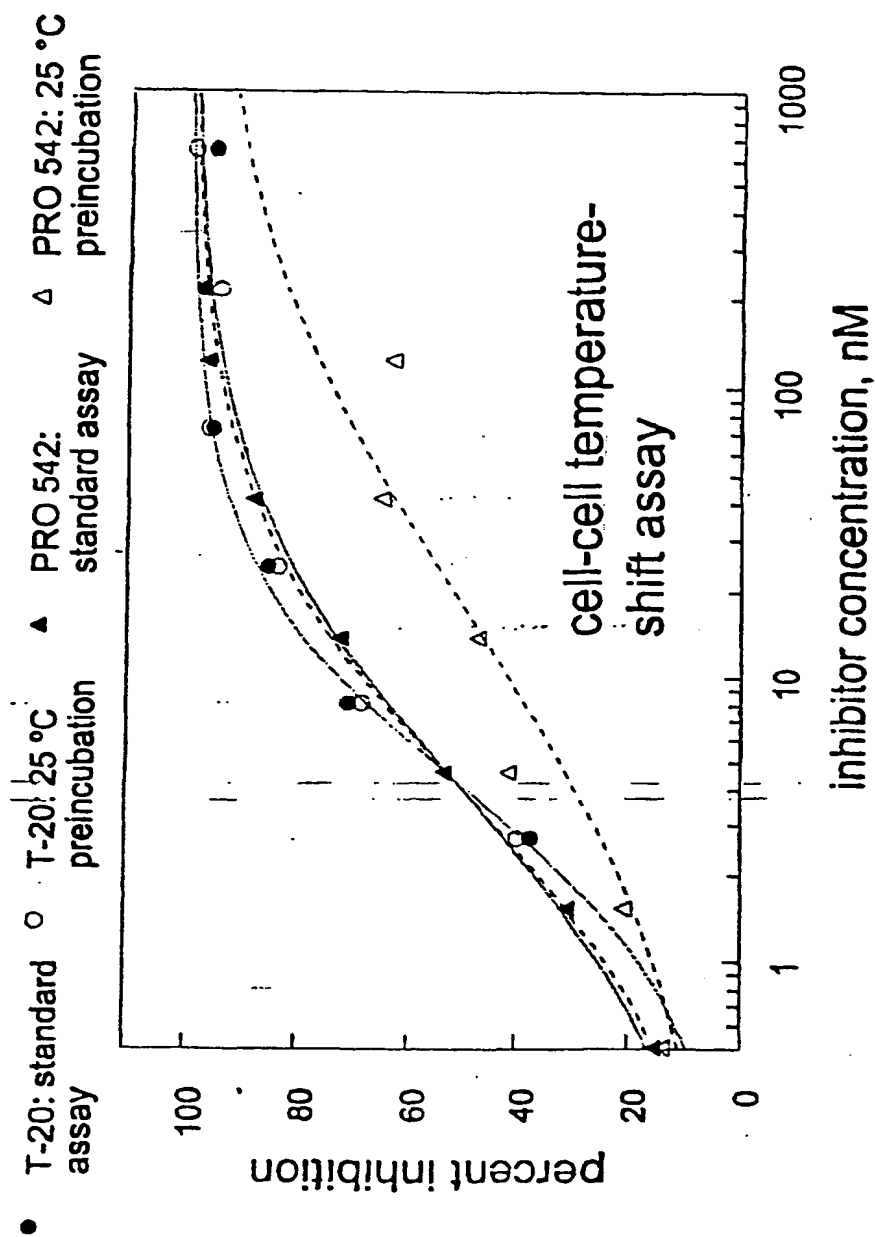*Formation of the Prehairpin Intermediate Requires CD4, Coreceptor and 37 °C (I)*



- T-20: standard assay
- ○ T-20: 25 °C preincubation
- ▲ PRO 542: standard assay
- △ PRO 542: 25 °C preincubation

Figure 25

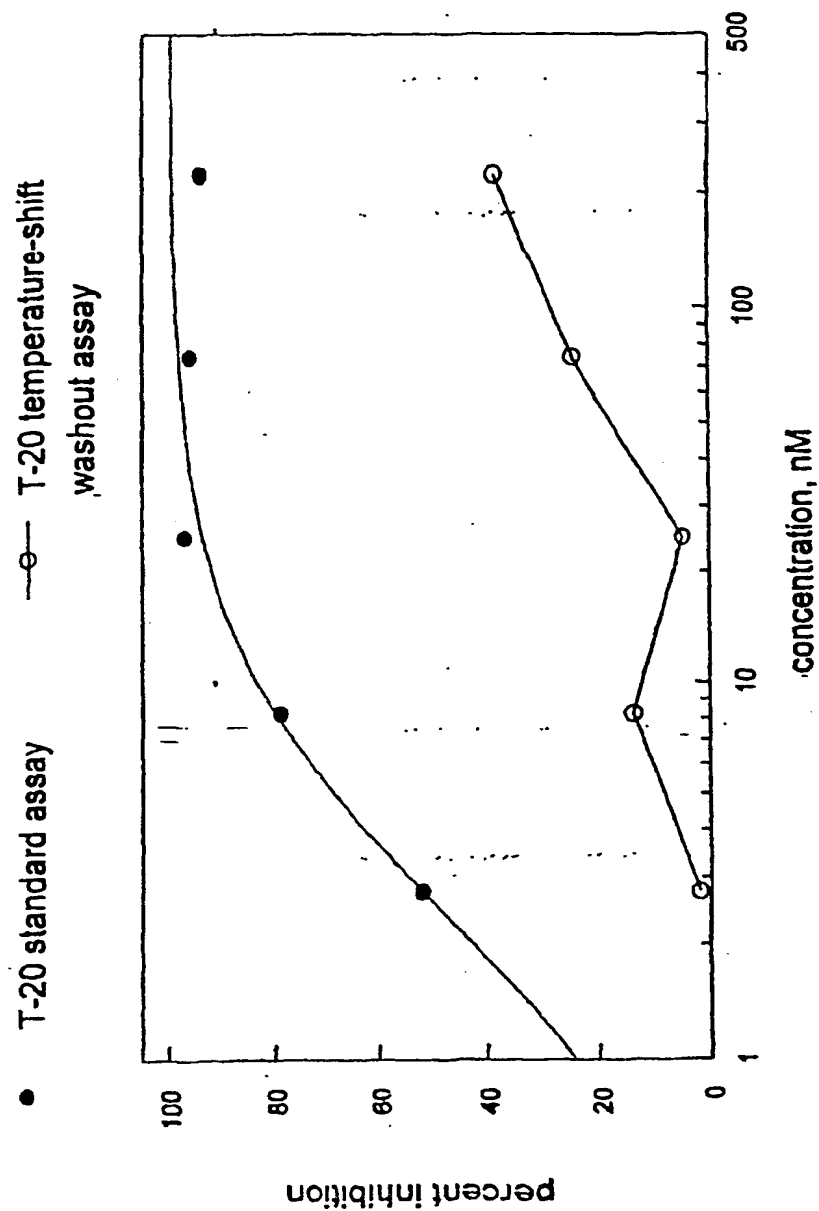## Formation of the Prehairpin Intermediate Requires CD4, Coreceptor and 37 °C (II)

Figure 26

# Possible Mechanism of Synergy: PRO 542
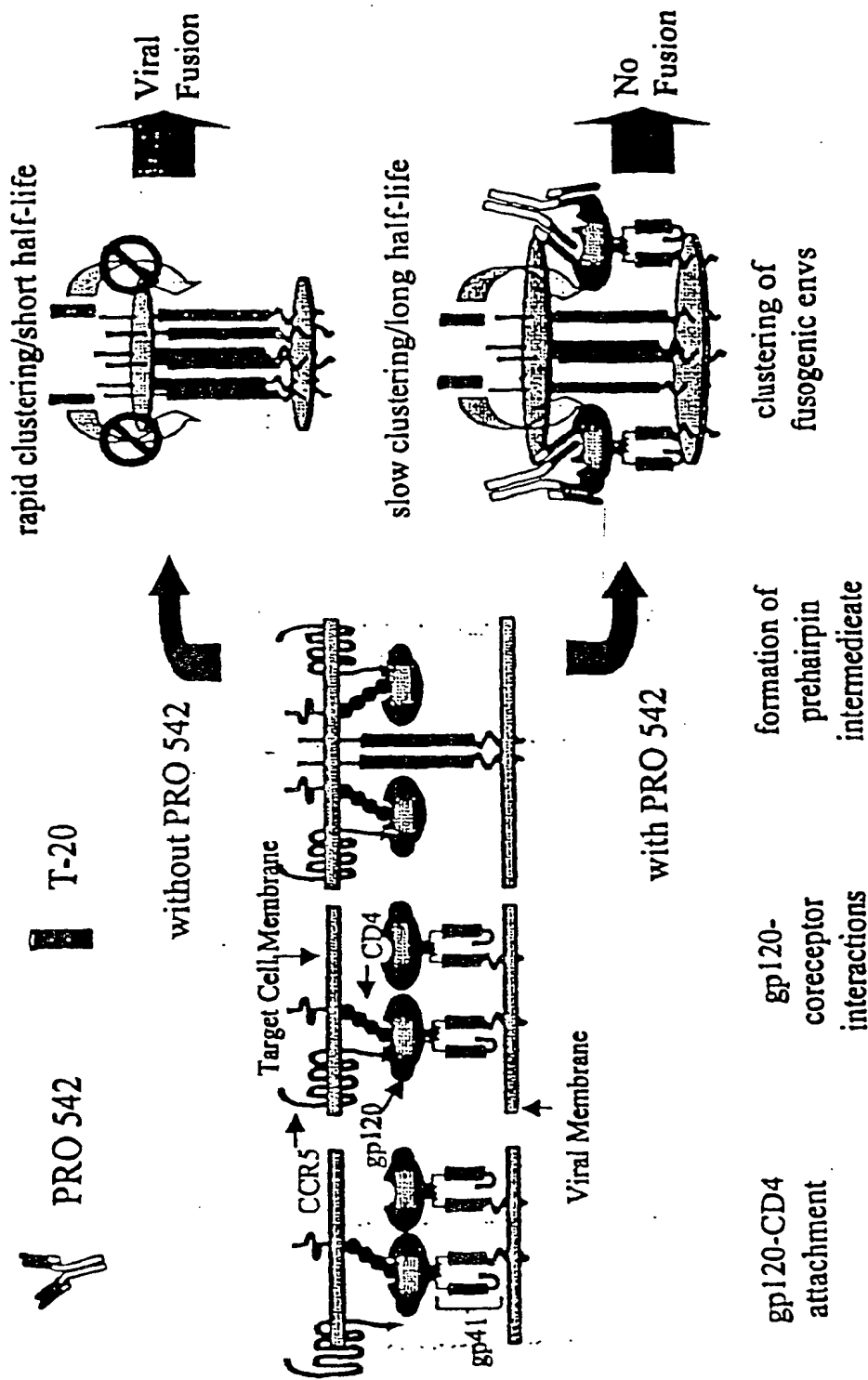# Increases the Half-Life of the T-20 Targets

Figure 27

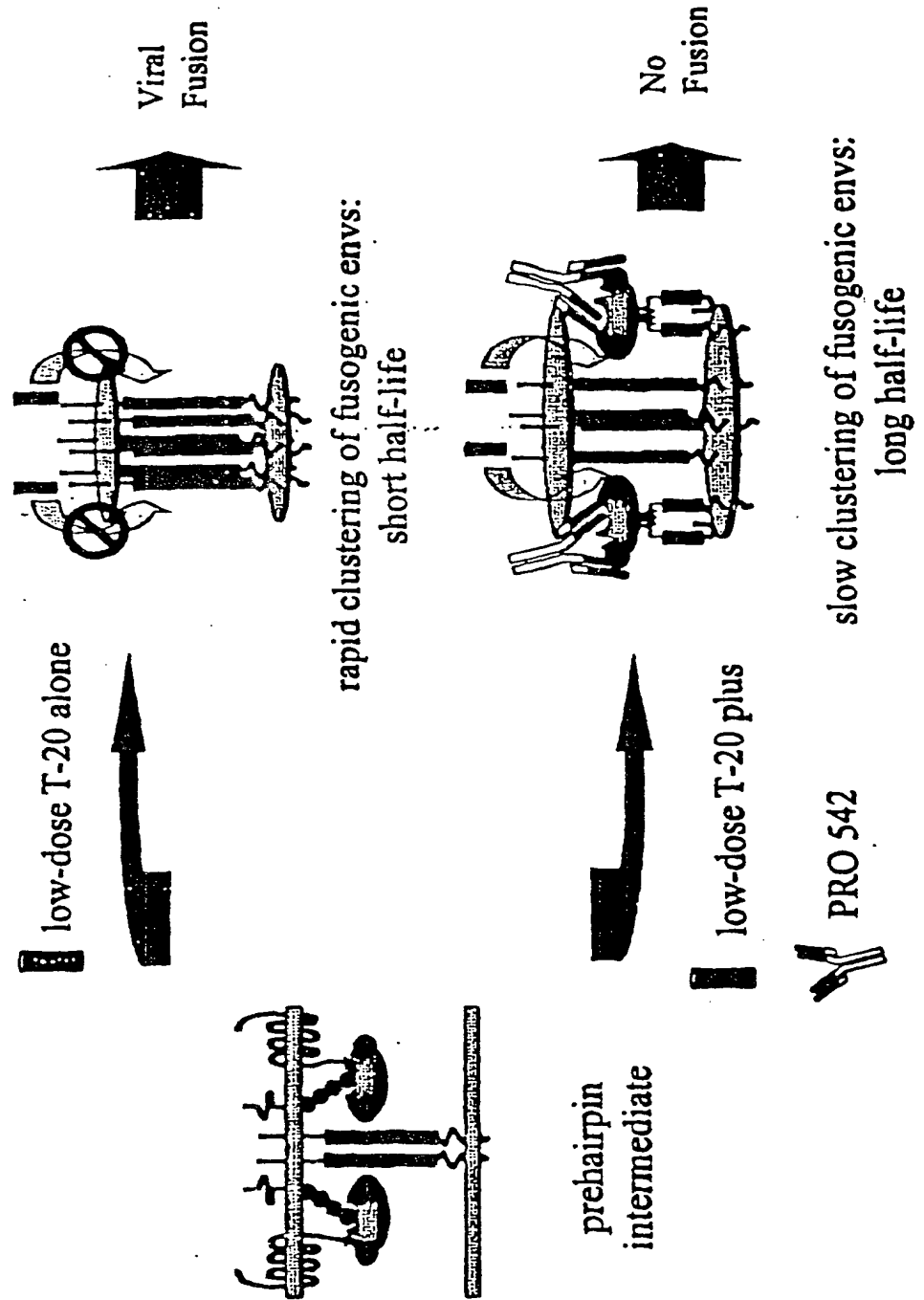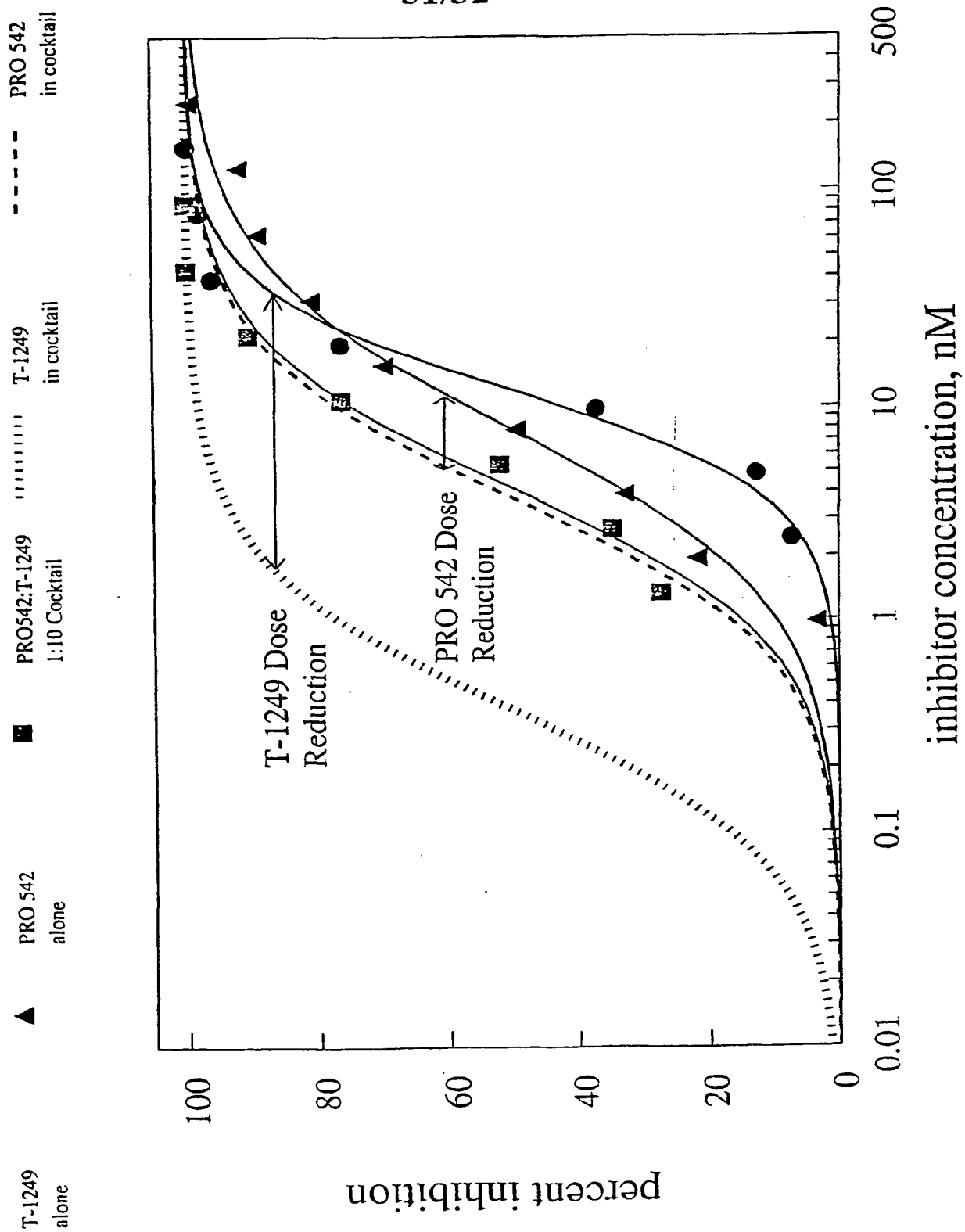# Possible Mechanism of Synergy: PRO 542 Increases the Half-Life of the T-20 Targets

Figure 28

Figure 29

| Fraction Inhibited | Dose PRO 542, nM (alone) | Dose PRO 542, nM (comb) | Dose T-1249, nM (alone) | Dose T-1249, nM (comb) | Combination Index | Dose Reduction PRO 542 | Dose Reduction T-1249 |
|---|---|---|---|---|---|---|---|
| 0.95 | 87.90 | 13.58 | 37.83 | 1.36 | 0.20 | 6.47 | 27.86 |
| 0.90 | 48.69 | 9.52 | 27.11 | 0.95 | 0.24 | 5.12 | 28.48 |
| 0.85 | 33.78 | 7.64 | 22.06 | 0.76 | 0.27 | 4.42 | 28.87 |
| 0.80 | 25.65 | 6.47 | 18.88 | 0.65 | 0.30 | 3.96 | 29.17 |
| 0.75 | 20.43 | 5.65 | 16.61 | 0.56 | 0.32 | 3.62 | 29.42 |
| 0.70 | 16.75 | 5.01 | 14.85 | 0.50 | 0.34 | 3.34 | 29.64 |
| 0.65 | 13.99 | 4.50 | 13.41 | 0.45 | 0.37 | 3.11 | 29.84 |
| 0.60 | 11.81 | 4.06 | 12.20 | 0.41 | 0.39 | 2.91 | 30.03 |
| 0.55 | 10.05 | 3.68 | 11.13 | 0.37 | 0.41 | 2.73 | 30.21 |
| 0.50 | 8.57 | 3.35 | 10.18 | 0.33 | 0.44 | 2.56 | 30.39 |

## SEQUENCE LISTING

<110> Progenics Pharmaceuticals, Inc.

<120> COMPOSITIONS AND METHODS FOR INHIBITION OF HIV-1 INFECTION

<130> 2048/62942-B-PCT

<140> Not Yet Known

<141> 2001-09-14

<160> 6

<170> PatentIn version 3.1

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<223> Description of Artificial Sequence: T20 Peptide

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Glu Lys Asn Glu Gln Gln Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu
            20                  25                  30


Trp Asn Trp Phe
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<212> PRT

<213> Artificial Sequence

<220>

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Thr Val Trp Gly Ile Lys Gln Leu Gln Ala Arg Ile Leu Ala Val Glu
            20                  25                  30


Arg Tyr Leu Lys Asn Gln
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<223> Description of Artificial Sequence: N34 Peptide

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Gln Gln His Leu Leu Gln Leu Thr Val Trp Gly Ile Lys Gln Leu Gln
            20                      25                      30

Ala Arg


<210>    4

<211>    28

<212>    PRT

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Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys
            20                      25


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            20                  25                  30

Ala Arg Ser Gly Gly Arg Gly Gly Trp Met Glu Trp Asp Arg Glu Ile
        35                  40                  45

Asn Asn Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn
        50                  55                  60

Gln Gln Glu Leu
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<223>   Description of Artificial Sequence: T-1249 Peptide

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1                   5                   10                  15

Ile Gln Gln Glu Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp
            20                  25                  30

Ala Ser Leu Trp Glu Trp Phe
        35